

**Effects of oxygen availability on hematological parameters,
immune status, gill histomorphology and gene expression of
Senegalese sole (*Solea senegalensis*): the role of acute
hyperoxia.**

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Abstract

Senegalese sole (*Solea senegalensis*) is a very attractive candidate for aquaculture due to its commercial value, despite its cultivation being hampered by different stressors and several infectious diseases responsible for high mortalities in its farming production. The low and high availability of oxygen occurring in the environment (named hypoxia and hyperoxia, respectively) is identified as a possible stressor for aquatic organisms, despite its consequences to Senegalese sole being still fairly unknown. Therefore, the present study aimed to assess the effects of oxygen availability in hematological parameters, immune status, gill morphology and gene expression on Senegalese sole. Fish around 30 g were exposed to different levels of dissolved oxygen for 4 and 24 hours. Mild hypoxia was established at 80% (negative control) while 2 hyperoxic conditions were tested (150 and 200%). Fish reared under normoxic conditions (100% dissolved oxygen) served as positive control. Supersaturation was obtained by injection of pure O₂ into the chambers, with a stable level of O₂ being kept in the chambers using an optimized oxygen regulator and analyzer. Following 4 and 24 hours fish were removed from the tanks and sampled to assess hematological (total and differential blood cell counts, hematocrit and hemoglobin) and innate immune (lysozyme, peroxidase) parameters as well as bactericidal activity, alterations in gill morphology and gene expression.

No mortalities were recorded during the course of the study. Regarding the hematological status of fish, hemoglobin increased significantly for the highest level of hyperoxia exposition tested (200%) after 4 and 24h exposure, while erythrocyte level was significantly higher for the 200% saturation after an exposition of 24h. Hematocrit (Ht) and red blood cells (RBC) levels were also higher for mild hypoxia when compared to normoxia.

Regarding the immune status, white blood cells (WBC) levels decreased for the 150% saturation after 4h of exposition and they also decreased for the 200% saturation after 24h of exposition compared to normoxia and mild hypoxia. Proportion of thrombocytes, lymphocytes, monocytes and neutrophils decreased at 200% saturation compared to normoxia and mild hypoxia, after 24h of exposition. Monocyte levels were also inferior for the same exposition period in the 150% saturation. When comparing exposition times, the 24h exposition to 200% saturation decreased in lymphocytes,

monocytes and neutrophils numbers when compared to the 4h exposition at the same saturation. Other immune parameters such as plasma lysozyme and peroxidase activities showed no significant alterations.

Glutathione peroxidase (GPX) expression in the head-kidney did not change among oxygen saturations and exposure time, suggesting there was no significant effect on this particular oxidative stress enzyme.

In summary, the present study suggests that 200% oxygen saturation presents an effect in hematological status with increasing red blood cells and hemoglobin after an exposition of 24 hours. Moreover, oxidative stress was not observed as indicated by no changes in GPX expression in fish exposed to hyperoxia. Thus, no negative conditions were recorded in this study after 24 hours of hyperoxia exposure. Nevertheless, further studies will be needed with higher exposition times and saturation levels tested to assess if hyperoxia can be truly beneficial and applied in fish farms in order to improve the conditions in which fish are hold.

Key-words: Senegalese sole; welfare; water oxygenation; hematology; immune responses; cell response; humoral parameters; oxidative stress

Resumo

O linguado senegalês é um forte candidato para aquacultura devido ao seu valor comercial. No entanto, o seu processo de produção é limitado por diferentes factores de stress e várias doenças infecciosas responsáveis por altas taxas de mortalidade. A elevada ou baixa disponibilidade de oxigénio que ocorre no ambiente (hiperóxia e hipóxia respectivamente) é identificada como um possível factor de stress para os organismos aquáticos apesar das suas consequências ao nível da produção do linguado senegalês serem ainda pouco conhecidas. Assim sendo, este estudo visou avaliar os efeitos da disponibilidade do oxigénio nos parâmetros hematológicos, estado imunológico, morfologia das brânquias e na expressão genética no linguado senegalês. Peixes com cerca de 30g foram expostos a diferentes níveis de oxigénio dissolvido durante 4 a 24h. O nível moderado de hipóxia foi estabelecido nos 80% (controlo negativo) sendo que duas condições de hiperóxia foram testadas (150 e 200%). Os peixes criados sob condições de normóxia (100% de oxigénio dissolvido) constituíram o controlo positivo. Foi obtido um estado de supersaturação através da injeção de O₂ puro nas câmaras de ensaio, mantendo um nível estável de O₂, usando um regulador e analisador de oxigénio optimizado. Após os intervalos de tempo de 4 e 24h, os peixes foram removidos das câmaras de ensaio e sujeitos à colheita de amostras para identificar parâmetros hematológicos (contagem total e diferencial de células sanguíneas, hematócrito e hemoglobina) e imunológicos inatos (lisozima, peroxidase), bem como a actividade bactericida, alterações na morfologia branquial e expressão genética.

Nenhuma morte foi registada durante a duração do estudo. No que respeita ao estado hematológico do peixe, a hemoglobina aumentou significativamente para o nível mais elevado de exposição à hiperoxia testado (200%) após uma exposição de 4 e 24 horas, enquanto que o nível de eritrócitos foi significativamente maior para a exposição de 200% após uma exposição de 24 horas. O Hematócrito (Ht) e níveis de eritrócitos (RBC) também foram mais elevados para o nível moderado de hipoxia quando comparado com normoxia.

Em relação ao estado imunitário, os níveis de glóbulos brancos (WBC) diminuíram para a saturação de 150% após 4 horas de exposição e também diminuíram para a saturação de 200% após 24 horas de exposição quando comparado com normóxia e nível moderado de hipóxia. A proporção de trombócitos, linfócitos, monócitos e neutrófilos diminuiu na saturação de 200% comparado com normóxia e nível moderado de hipóxia, após 24 horas de exposição. Os níveis de monócitos também foram inferiores para o

mesmo período de exposição na saturação de 150%. Aquando da comparação de tempos de exposição, a exposição de 24 horas à saturação de 200% causou um decréscimo nos números de linfócitos, monócitos e neutrófilos quando comparado com a exposição de 4 horas à mesma saturação. Outros parâmetros imunitários como a actividade da lisozima presente no plasma e a actividade da peroxidase não mostraram quaisquer alterações significativas.

A expressão de Glutathione peroxidase (GPX) no rim anterior não sofreu alterações nas saturações de oxigénio testadas e durante o tempo de exposição, sugerindo não haver um efeito significativo nesta enzima de stress oxidativo.

Em suma, o presente estudo sugere que, em relação ao estado hematológico, uma saturação de 200% de oxigénio provoca um aumento de células sanguíneas e de hemoglobina após uma exposição de 24h. Mais ainda, não foi observado stress oxidativo como indicado pela ausência de alterações na expressão de GPX dos peixes expostos a hiperóxia. Assim, não foram encontradas alterações negativas após 24h de exposição a hiperóxia. No entanto, serão necessárias mais investigações que testem tempos de exposição mais prolongados e maiores níveis de saturação de oxigénio para avaliar se a hiperóxia poderá ser verdadeiramente benéfica e aplicada nas pisciculturas, de modo a melhorar as condições a que os peixes estão sujeitos.

Palavras-chave: Linguado senegalês; bem-estar; oxigenação da água; hematologia; resposta imunitária; resposta celular, parâmetros humorais; stress oxidativo

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Introduction

World Aquaculture

Aquaculture is defined as the science and technology related to production of aquatic species, with its main purpose being an increase in production that surpasses the natural capacities of these species (White et al., 2004). Due to the combined effects of overfishing, pollution, habitat destruction, ecological and biotic changes caused by global warming, as well as many other human activities, the ocean resources are being depleted at an alarming rate (White et al., 2004). This includes stocks of marine species that are being exploited and are very close to their maximum sustainable limits. Thus, aquaculture is important to improve and create additional means of producing fish and serve as a supplement to the production of wild capture fisheries (White et al., 2004). Aquaculture may therefore serve as an essential provider and supplier of food to an ever-growing demanding population and increase its importance even more in the nearby future.

World's fish consumption *per capita* has increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 (FAO, 2014). Following up to this, fish production has been increasing during the last decades, and according to recent studies the demand for more production will not stop growing. This increase is mainly associated with population growth, better incomes, urbanization, an improvement in the distribution channels and the worldwide expansion of fish production (Imsland et al., 2003). Developed countries are still the ones that consume more fish as of 2014 although fish consumption in developing countries is clearly improving and straightening the distance to developing countries (FAO, 2014). Total world capture in 2011 was of 93.7 million tons, reaching the second highest ever value, only below the 93.8 million tons produced in 1996. In 2012 it was also registered a new maximum production total of 86.6 million tons (FAO, 2014).

In 2010 fish protein accounted for about 16.7 percent of total animal protein intake by the world population and 6.5 percent of total protein consumed. These stats demonstrate how important fish proteins can be in the diet of millions of people, especially in overly populated areas (FAO, 2014).

Aquaculture and fisheries production has reached a total of 158 million tons, in 2012, with 136 million being entirely for human consumption (Figure 1 and 2). Global aquaculture production continues to expand and has reached an all-time gross of 90.4

million tons, consisting 66.6 million of food fish and the other 23.8 million of aquatic algae, in 2014, which roughly estimates in 144 billion dollars. The global fish aquaculture has continuously been improving, with an expansion of 49.9 million to 66.6 million tons only in the 2007-2012 period (Figure 1). Between 2000-2012, world aquaculture production has had an average annual rate growing of 6.2% (FAO, 2014).

	2007	2008	2009	2010	2011	2012
<i>(Million tonnes)</i>						
PRODUCTION						
Capture						
Inland	10.1	10.3	10.5	11.3	11.1	11.6
Marine	80.7	79.9	79.6	77.8	82.6	79.7
Total capture	90.8	90.1	90.1	89.1	93.7	91.3
Aquaculture						
Inland	29.9	32.4	34.3	36.8	38.7	41.9
Marine	20.0	20.5	21.4	22.3	23.3	24.7
Total aquaculture	49.9	52.9	55.7	59.0	62.0	66.6
TOTAL WORLD FISHERIES	140.7	143.1	145.8	148.1	155.7	158.0
UTILIZATION¹						
Human consumption	117.3	120.9	123.7	128.2	131.2	136.2
Non-food uses	23.4	22.2	22.1	19.9	24.5	21.7
Population (<i>billions</i>)	6.7	6.8	6.8	6.9	7.0	7.1
Per capita food fish supply (<i>kg</i>)	17.6	17.9	18.1	18.5	18.7	19.2

Figure 1 - World fisheries and aquaculture production and utilization, Source: FAO (2014)

About 10 to 12% of the world's population depends on the fisheries and aquaculture activity to sustain their livelihood (FAO, 2014). Fish continues to be one of the most traded commodities worldwide being of special importance to developing countries and, in some cases, accounting for more than half of the total traded commodities by these countries (FAO, 2014). In 2012, 10% of the total agricultural exports corresponded to fish and related fishery products. Although developing countries continue to dominate worldwide trade of fishery products, in recent years their dominance has been decreasing. This can be explained by the rapid rising of developing countries in the worldwide fish trade during the recent decades (FAO, 2014).

World aquaculture production is separated in 2 types, inland aquaculture and mariculture. Inland aquaculture normally uses freshwater albeit there are production operations that use saline water. Mariculture is related to productions operations in the sea as well as land-based production facilities. Inland aquaculture growth has surpassed

mariculture growth, being the main contributor to the total food fish production worldwide (Sardinha, 2009; FAO, 2014).

From the organisms produced in aquaculture, freshwater fish dominate the world production with about 56.4% (approximately 33.7 million tons), followed by molluscs with 23.6% (14.2 million tons), crustaceans with 9.6% (5.7 million tons), diadromous fish with 6.0% (3.6 million tons), marine fish with 3.1% (1.8 million tons) and other aquatic organisms with 1.4% (814 300 tons) (FAO, 2014).

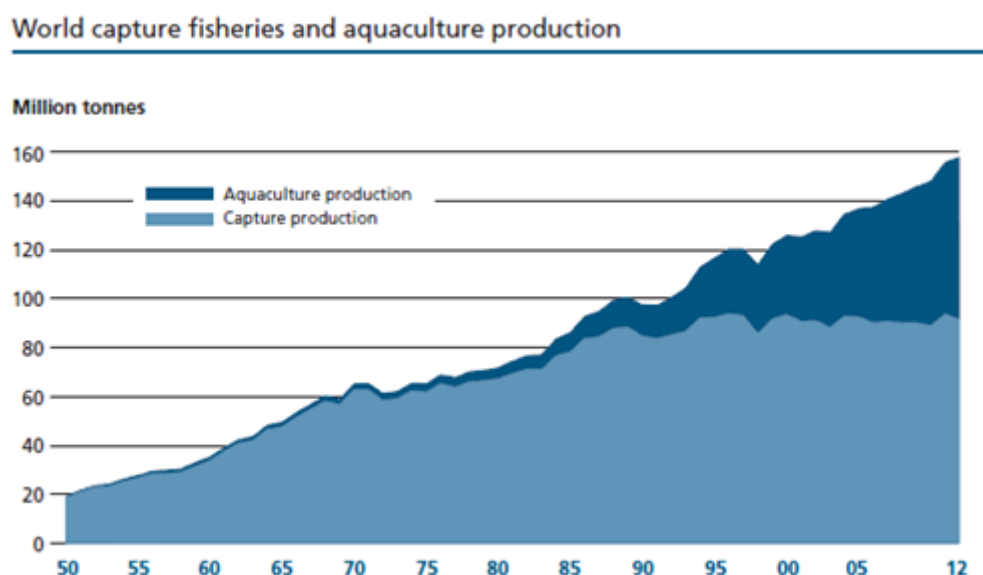


Figure 2 – World capture fisheries and aquaculture production, source: FAO 2014

Aquaculture in Portugal

Located in the region further west of the Iberian Peninsula and bordered by the Atlantic Ocean to the north and west, Portugal is the most western country in Europe. The Algarve region at the far east of the south coast also exhibits a strong influence from the Mediterranean Sea. Although Portugal is a small country, with only 88.700 km² area, its mainland stretches along 780 km of the Atlantic coastline (JNICT, 1990). In spite of its small area it has two major climate systems. The north side of Portugal is a colder, mountainous region comprising most of the country's rivers and reservoirs more suitable for the production of cold water species such as rainbow trout (*Oncorhynchus mykiss*) and flatfish (turbot- *Scophthalmus maximus*; Senegalese sole– *Solea senegalensis*) (Gouveia, 2014). The center and specially the south of the country (the Alentejo and Algarve regions) contain a drier, warmer plain, more suitable for the production of warmer water species due to the influence of the Mediterranean Ocean. These include European sea

bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*), meagre (*Argyrosomus regius*), red porgy (*Pagrus pagrus*) and white sea bream (*Diplodus sagrus*) (Gouveia, 2014).

Nowadays, the Algarve region is the most important Portuguese marine aquaculture region, comprising 45 percent of national production (INE, 2011). Besides the Portuguese mainland, Portugal also includes the Azores archipelago (2.344 km²) and the island of Madeira (796 km²), both located in the Atlantic Ocean, 1380 km from the Portuguese coast and 660 km from the West African Coast, respectively (JNICT, 1990). Madeira has a significant on-growing marine fish production industry, mainly farming gilthead sea bream (Gouveia, 2014).

Portugal as an aquaculture producer has a great potential, being considered an European potency. Due to its great exposition to the sea, having a great coastal area as well as excellent ambient conditions, Portugal is quite privileged geographically speaking. In part owing to the incoming water from the Mediterranean Sea, Portugal tends to have water at moderate temperatures (varying between 13° C and 18° C). According to the reasons above, Portugal has more than enough proper conditions for the aquaculture production of a number of species with economic value (Sardinha, 2009; FAO, 2014).

One of the things that also make Portugal so attractive for the aquaculture activity is the fact that Portugal tends to rate quite high in the overall world fish consumption placing very close to countries like Japan (the main consumer of fish in the world). The fact that the national fishing activity has stagnated, and having in account the overall country's fish consumption, it is of great importance to boost aquaculture activity in Portugal, and in this manner create alternatives to the traditional ways of fish supplying (FAO, 2014). Still, Portugal is lacking in some things that could, otherwise, make aquaculture activity way easier to be established. The main reasons for this are related to bureaucracy and legal problems, with the establishment of facilities being quite hard owing to the long legal processes until the obtainment of permission (Sardinha, 2009).

Senegalese sole

The Senegalese sole, *Solea senegalensis* (Kaup, 1858) (Figure 3) is a flatfish with oval and asymmetric body (eyes on the right side). It is commonly found in the Atlantic, from the Gulf of Biscay to the coasts of Senegal. It can also be found, more rarely, in the western Mediterranean. This species is very similar to the common sole, *S. solea*, a species whose distribution is more to the north, from the North Sea to the Moroccan coast. It is very difficult to distinguish larvae between both species (Lagardère et al., 1979).

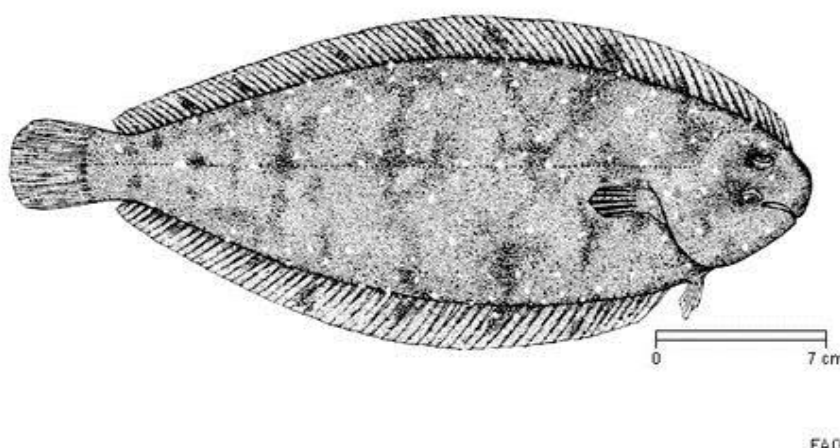


Figure 3 – *Solea senegalensis* Source: FAO (2012)

It is a benthonic marine species living in sandy or muddy bottoms, off coastal areas up to 100 meters depth. It feeds mainly on benthonic invertebrates, like for example larvae from polychaetes and bivalve molluscs. It may occasionally feed on small crustaceans. Studies made to analyze stomach contents showed a prevalence of polychaetes, but amphipods, copepods and isopods were also found (Drake and Arias, 1989).

This species is gonochoric, with females reaching maturity at around 3 years, with a close size to 32 cm (Ramos, 1982). It naturally spawns from March to June (Ramos, 1982). When it reaches sexual maturity the growth rate decreases dramatically (Dinis, 1986).

Senegalese sole is more commonly found in warm climates than its relative common sole, being amply raised in semi-extensive earthen ponds and land-based facilities in Southern Spain (Rodriguez, 1984; Manzano, 1993; Dinis et al., 1999) and Portugal (Dinis, 1986; Pousão-Ferreira, 1993; Dinis et al., 1999). In Portugal it is also being explored intensively in the northern region (Ramalho and Dinis, 2010).

Senegalese sole growth in captivity is quite satisfactory (Olmedo et al., 2003; Rodriguez and Souto, 2003) and hatchery reproduction has been improving (Anguís and Cañavate, 2005; García Lopez, 2006). Adding to this, larval rearing and weaning are not problematic issues (Salas-Leiton, 2009).

According to the reasons cited, Senegalese sole is a very attractive candidate for marine aquaculture and has a very big potential for future farming at commercial scale. However, despite the constant increase of sole production in both Portugal and Spain, there are still unresolved problems with its production, its cultivation being hampered by its high sensitivity to different stressors and to several infectious diseases that can cause large mortalities (Imsland et al., 2003; Costas et al., 2008). Abnormal pigmentation and morphological malformation is another current issue and, while the fish is perfectly healthy, apart from these malformations, marketing will be more difficult due to the strange appearance of the fish (Dinis, 1996). Adding to this, growth and survival from juvenile to market-size fish is not fully controlled with regard to rearing technology and husbandry conditions, feeding behavior and nutritional requirements.

Aquaculture concerns in Senegalese sole

The environment in which an animal grows will impact its physiology and behavior in several ways, both in a negative and positive manner. So it is of vital importance to know how the aquaculture systems may affect the fish in numerous ways. It has been proven that factors such as light, salinity, temperature, pH and oxygen should be manipulated very carefully so that fish may have a proper and healthy development (Bowden, 2008). If by any chance, one of these factors is not well controlled, stress will be induced into the fish. Depending on the type of stressor, as well as the duration of the exposure and its intensity, the out-coming result may go from a quick recovery to everlasting imbalances of the fish organism and, in more severe cases, up to the death

(Bowden, 2008). Other stress inducers also arise due to high stocking density (Costas et al., 2008), handling (Aragão et al., 2008) and confinement of the fish (Tort, 2011). Since these factors may have negative impacts to the fish well-being and development, it is of vital importance to apply good management practices in fish farms, to minimize potential economic losses that could arise from stress effects.

It has been shown in several studies that after animals are subjected to stress, the chances of being afflicted by diseases increase significantly. This can be explained by the allocation of resources to face the stress-inducing challenge, leaving the immune system in a weaker, less efficient state, and therefore more susceptible to pathogens (Ellis, 2001; Tort, 2011). Depending on the nature of the stressor, i.e. acute or chronic, the consequences may lead to different responses (Figure 4). In a fish farm, especially if it uses intensive recirculating aquaculture systems, fish have higher chances of suffering from chronic stress, owing to environmental (water quality and hypoxia) and health conditions (diseases and parasites) (Oliva-Teles, 2012). Chronic situations such as those, where the intensity of the stressor is low but persistent, are not very common in nature, yet these situations are normally found in fish subjected to anthropogenic activities like pollution and aquaculture (Tort, 2011). Chronic stress tends to be more exhausting to the organism, as it is normally associated with longer exposition, making it more demanding to the organism in terms of resources and energy spent to cope with it.

In fish, the well-known negative effects of stress on the immune system are associated to maladaptive responses to chronic stressors (Tort, 2011). For example, cortisol - used as a general indicator of stress conditions in teleost fish (Mommsen et al., 1999), since its plasma levels increase significantly in response to several stressors (Acerete et al., 2004) - may decrease the number of lymphocytes, suppress phagocytic and complement activities in head-kidney and blood as well as increase susceptibility to infection (Ortuño et al., 2001). Nevertheless, acute cortisol levels may also induce the immune system to react more rapidly and effectively to possible consequences from a stressor, improving its adaptive function to environmental stressors (Verburg-van Kemenade et al., 2009).

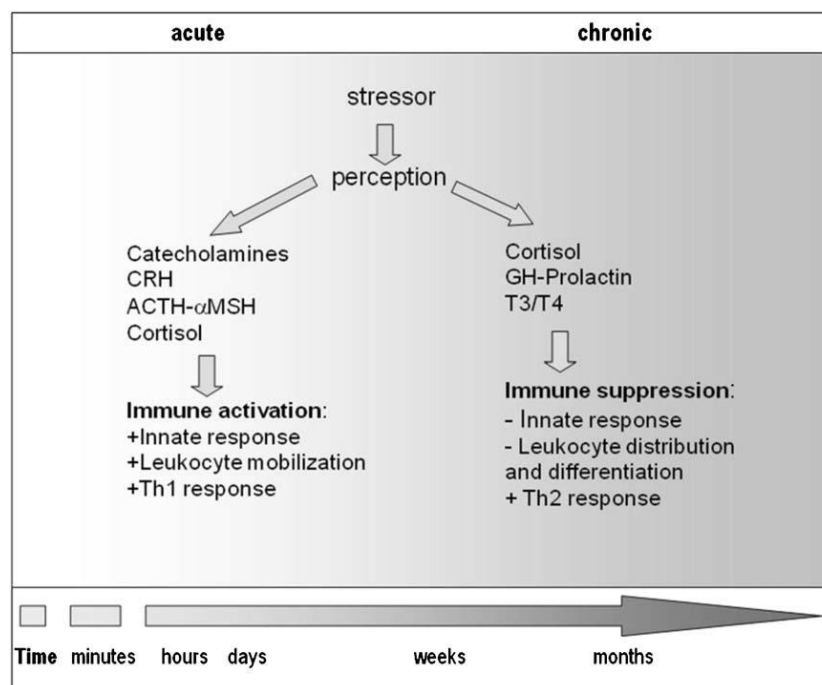


Figure 4 – Stress and immune response, Source: Tort, 2011

Immune System of fish

For multicellular organisms such as fish, effective mechanisms are needed to combat all kinds of pathogens encountered in the environment. This is done through the immune system. According to the complexity of the organism and its necessities, the immune system is divided into innate and specific. On primitive multicellular organisms one will only find the innate immune system, while in more complex organisms both the innate and acquired immune systems are present – this includes all species of fish, even the more primitive ones like lampreys (Tort, 2011).

The innate immune system is characterized by a more rapid and nonspecific response, being divided in physical barriers, cellular and humoral components (Whyte, 2007). It is the first line of defense against possible infections having a complex connection with the specific immune system. Signals emerging due to cell stress and tissue damage will activate the innate immune system and, using co-stimulators, it will proceed to activate and determine the nature of the specific response (Magnadottir, 2006; Muiswinkel, 2014). Another very important function is the maintenance of homeostasis of the organism, keeping a cellular and molecular equilibrium. This is accomplished during the renewal and maintenance immediately after injuries or inflammatory responses taken by the body, as well as during natural processes of cell death (Magnadottir, 2006).

The specific (also known as adaptive) immune system is primarily composed of highly specialized cells and processes that recognize specific antigens through the so-called antigen presentation, causing responses to eliminate specific pathogens and infected cells (Janeway et al., 2005; Rauta et al., 2012). One important aspect of the specific immune system is the acquiring of immunological memory, in which, after contact with a specific pathogen and subsequent response, all further encounters with the same pathogen will lead to an increase of the effectiveness of the immunological response (Janeway et al., 2005; Rauta et al., 2012). The immune system, thanks to this process, will be prepared for future infections caused by the same pathogen, unleashing a way quicker and more effective response (Parkin, 2001; Rauta et al., 2012).

Although the innate response is faster than the specific one, it can lead to the damage of own healthy tissues, due to a lack of specificity. This will not happen in the specific response, since it is a quite precise mechanism (Magnadottir, 2010). However, acquired immunological mechanisms are highly complex, specific and present great diversity and memory, making specific immunity biologically costing and time-consuming, taking days or even weeks to reach its terminus (Parkin, 2001; Rauta et al., 2012).

The innate immune system encompasses a wide range of different defense mechanisms, each with its own singular purpose. Epidermis, fish scales and the mucous from the skin constitute the physical barriers and first line of defense against pathogens (Ellis, 2001; Magnadottir, 2006; Whyte, 2007). Mucous, in particular, has been widely studied, and aside from the obvious involvement of pathogens and its slowing of pathogen propagation, it also has a lot of components that have antimicrobial properties, such as lysozyme, lectins, antibacterial peptides among others (Whyte, 2007; Magnadottir, 2010). If these barriers are overcome by pathogens, there are still immune mechanisms to neutralize these threats, for instance cellular defenses, humoral factors and inflammation process (Whyte, 2007; Magnadottir, 2010; Muiswinkel, 2014).

The cellular defenses of the immune system encompass a myriad of different cell types with the most distinguished ones being leucocytes and thrombocytes (Ellis, 2001; Magnadottir, 2006). Thrombocytes' function is to stop bleeding by means of blood clotting, gathering at the injury site and plugging the hole. In fish they are complete cells (opposing the mammal platelets that are only a fragment of cells). Their shape is typically fusiform but can vary (Esteban et al., 2000; Robert and Ellis, 2012). Leucocytes are divided in several different types involved in both the innate and adaptive response. These include monocytes/macrophages and granulocytes, such as neutrophils, and lymphocytes (Secombes, 1996; Ellis, 2001, Magnadottir, 2006).

Cellular defenses from the innate arm of the fish immune system include the phagocytic cells (neutrophils and macrophages) – these produce cell compounds with antimicrobial properties activated during the inflammation process -non-specific cytotoxic cells, endothelial cells and dendritic-like cells (Evans *et al.*, 1992; Secombes *et al.*, 1996; Dalmo *et al.*, 1997; Whyte, 2007; Muiswinkel, 2014). Phagocytic cells act primarily to prevent or mitigate the initial spread of the infectious organism and its growth (Magnadottir, 2006; Neumann *et al.*, 2011). Macrophages are typically mononuclear and normally peroxidase negative, being present in all body cavities (Secombes, 1996). Neutrophils, on the other hand, are normally polymorphonuclear, being encountered in blood and in a reserve pool in the hematopoietic organs, and more scarcely, in tissues and body cavities (Afonso *et al.*, 1998; Secombes, 1996). Neutrophils are identified due to their granules staining in reaction to specific dyes and enzymes, such as peroxidase (Afonso *et al.*, 1998). Both these phagocytic cells are responsible for a process called phagocytosis that includes chemotaxis, attachment, ingestion, degranulation, intracellular killing and intracellular digestion (Parkin, 2001). One important reaction that takes place during this process is known as respiratory burst, which basically degrades pathogens and destroys bacteria through the release of reactive oxygen species and nitric oxide (NO), since these are powerful antimicrobial agents (Whyte, 2007; Costas *et al.*, 2013; Muiswinkel, 2014).

Humoral factors relates to all kind of macromolecules responsible for immunity that are present in body fluids (Yano, 1996). Humoral factors are present both in the adaptive and innate immune system. For the innate system, the humoral factors consist of complement proteins (biochemical cascade with several pathways, responsible for complementing phagocytic cells in the elimination of pathogens) (Ellis, 2001) and antimicrobial peptides as well as other components also found in these extracellular fluids, such as lysozyme and interferons (Bols, 2001; Whyte, 2007). For instance, lysozyme is an enzyme found commonly in tissues and fluids of fish, particularly the mucous, acting on the walls of Gram-positive bacteria damaging them (it acts on the peptidoglycan layer of the wall disrupting the glycosidic bonds) (Bols, 2001; Ellis, 2001). Interferons are essential in the host immune defense against viruses, bacteria, parasites and tumor cells, being capable of inducing apoptosis and inhibiting cell proliferation (Samuel, 1991; Bols, 2001; Ellis, 2001; Seppola *et al.*, 2007; Whyte, 2007; Secombes, 2011).

Effects of hypoxia and hyperoxia in fish

When a body is not supplied with enough oxygen - having in that case a low oxygen level in its organic tissues - it is considered that the body suffers from the pathologic condition named hypoxia. Although there are many definitions of this condition, the more widely used is the one that considers any level of dissolved oxygen that is low enough so as to cause negative effects on the physiology and behavior of an organism (Boleza et al., 2001). Hypoxia naturally occurs in aquatic systems, being caused by a great number of factors, for example pollution, eutrophication, natural phenomenon like photoperiod and temperature, water flow rate, etc. (Boleza et al., 2001).

The availability of oxygen in water is one of the most important water quality parameters in aquaculture (Timmons et al., 2001). Most of modern day aquaculture facilities produce fish at high densities and, since oxygen consumption is way higher than normal, the oxygen provided to the system may not be enough to supply all the fish with oxygen, thus they will start suffering from hypoxia. If this is left unchanged high mortalities will naturally ensue (Bunch and Bejerano, 1997).

Furthermore, hypoxia facilitates the susceptibility of fish to pathogens, as has been shown in several studies made with tilapia and carps where hypoxia levels contributed to an increase of infection with *Streptococcus sp.* (Bunch and Bejerano, 1997; Boleza et al., 2001). Also, outbreaks of diseases occur more easily, as is the case with mycobacteriosis in fish (Vogelbein et al., 1999). There is an increase of carbon dioxide pressure in the organism due to respiration, which typically leads to respiratory acidosis (low pH), as the level of dissolved oxygen is too low to maintain an equilibrium, following up to the increasing carbon dioxide in the system (Bunch and Bejerano, 1997). If exposed to hypoxia conditions, an increase of ammonia toxicity naturally follows as was proven in studies with freshwater fish, *Sparus aurata* and turbot (*Scophthalmus maximus*) (Thurston et al., 1981; Wajsbrot et al., 1991; Person-Le Ruyet et al., 1997). Exposure to hypoxia causes an increase in hemoglobin concentration and erythrocyte number. Hemoglobin is also capable of binding oxygen in a more effective way. Hypoxia induces other type of physiological changes, such as increasing gill diffusion and increasing the gill surface area so that the oxygen levels can be maintained at an appropriate level (Randall and Kuiter, 1982; Randall and Taylor, 1988; Wu and Woo, 1984).

Hypoxia seems to affect negatively the immune system of organisms, limit and in more severe cases shut down mechanisms of the immune system. One of these cases is the respiratory burst of phagocytic cells (Feldman et al., 1990). Moreover, the complement

system may also be suppressed but the reports are still conflicting on this case, as one particular study showed a decrease in the complement haemolytic activity on channel catfish, yet other studies showed no changes to the complement system (Ortuño et al., 2002; Welker et al., 2007).

While hypoxia is associated with low oxygen levels in the water, the opposite case also exists, and is defined as hyperoxia – an excess of oxygen in body tissues or in the environment (Mach et al., 2011).

Having in consideration that many fish farms need to concern with the levels of oxygen - due to increasing stocking densities, deficient water supply and organic waste - inducing these systems with oxygen supplementation may in fact benefit fish (Person-Le Ruyet et al., 2002). Many factors have to be taken in consideration to determine the optimum level of O₂ saturation for farmed fish, the most important ones being growth rate and the cost of O₂ (Crampton et al., 2003). If a farming facility is capable of maintaining the oxygen saturation at an optimum level that leads to an increase in the growth rate while not consuming too much oxygen, production as well as income will augment significantly (Bergheim et al., 2005). One way of improving saturation levels is by increasing water exchange rate, adding O₂ in the water or simply by reducing the biomass of the rearing units. However, supplementing oxygen to the water, spending more energy to improve water exchange and reducing stocking density will affect the total income of the facility. All these variables incur on higher costs due to increased use of energy and oxygen, or because of reduced productivity as biomass is decreased (Bergheim et al., 2005).

Some farming industries, such as the salmon one, are known for supplementing oxygen to supersaturate the water with O₂, as farmers believe it will improve the potential growth and even increase the pathogen resistance in salmons (Calldwell and Hinshaw., 1994). There are still doubts as to what extent and how, especially on the long term, the supersaturation of oxygen affects fish (Person-Le Ruyet et al., 2002). And while there are some studies that tried to indulge on this subject, the conclusions were not sufficient to support the idea of O₂ supersaturation enhancing growth and improving the immune system. Some evidence does exist that moderate hyperoxia can improve growth of fish as was proven in some studies (Sargolia et al., 1995; Foss et al., 2002; Hosfeld et al., 2008), but still other studies failed to prove any improvement in the growth of fish under hyperoxic conditions when compared with normoxic conditions (Edsall and Smith, 1990; Calldwell and Hinshaw, 1994; Person-Le Ruyet et al., 2002). Growth in other flat-fish has also been studied but no significant growth was noticed, as was the case with turbot

exposed to 147% and 223% saturation compared with normoxic conditions (Person-Le Ruyet et al., 2003). Also, ammonia tolerance can be improved in rearing fish by increasing oxygen saturation above normoxia, as was proven with spotted wolfish (Foss et al., 2003).

Nevertheless, supersaturation can still be harmful to fish depending on the concentration of O₂ and the duration of exposition, and in more severe cases even cause mortalities (Calldwell and Hinshaw., 1995; Lygren et al., 2000; Ritola et al., 2002; Fridell et al., 2007). One needs to have in consideration the toxicity of oxygen and how it may vary depending of the species, life stage and overall conditions in which the animals are kept, as a hyperoxic threshold exists for every single organism (Colt and White., 1991).

The physiological effects caused by hyperoxia are a decrease in breathing due to decreased ventilation frequency that leads to accumulation of CO₂ in the blood causing respiratory acidosis (Dejours, 1977), imbalance in gill ion concentrations induced by respiratory acidosis (Brauner et al., 2000), as well as reduced branchial chloride cell fractional surface area (Goss et al., 1994). In some cases, during transportation, pure O₂ is added to the water and some negative effects are registered such as plasma ion imbalance, hypercapnia, respiratory acidosis and stress in Atlantic salmon smolts (Brauner et al., 2000). Other kind of disturbances are also observed when measuring stress indicators, with effects on hematocrit, hemoglobin, osmolality, cortisol, lactate and glucose (Calldwell and Hinshaw., 1995), and plasma chloride (Brauner et al., 2000). Immunologically, hyperoxia was found to increase the antibody levels in a study developed on the effects of water oxygenation on the immune system of the European sea bass, as higher concentrations of antibodies were present in the sera of fish held under hyperoxic conditions than in the sera of fish held under normoxic conditions (Mazzini et al., 1998), although more studies are needed to discover how fish may benefit from this, since there is still a lack of information about the influence of oxygen concentration on the antibody response in fish (Bowden, 2008).

Oxidative stress

Exposure to hypoxia and hyperoxia can be potentially dangerous to aquatic organisms such as fish, causing overall damages to the organism limiting its growth and causing a lower biomass production, aggravating therefore aquaculture production (Wedemeyer, 1997). The exposure to hyperoxia causes a reduction of gill ventilation and an elevation of the partial pressure of CO₂ in blood, contributing to respiratory acidosis. While respiratory acidosis can easily be nullified or compensated within days, the major problem derives from gill oxidative damage (Brauner et al., 2000). The extreme ambient conditions of both hypoxia and hyperoxia may cause serious damage in tissue lipids, proteins, DNA and other key molecules (Kregelet Zhang, 2007; Kowaltowski et al., 2009). Damages are mostly done by reactive oxygen species (ROS), these being essentially by-products of the reduction of O₂ into energy and water. ROS, such as superoxide radical, hydrogen peroxide and hydroxyl radical are known to cause oxidative stress in fish in case the antioxidant defenses are overwhelmed and cannot fight back the pro-antioxidant forces, leading to changes in molecular and membrane structures that end up causing cell and tissue damage (Reznick et al., 1998). These antioxidant forces, protecting the aquatic organisms, refer mainly to antioxidant enzymes and low molecular weight scavengers. Antioxidant enzymes may therefore serve as possible markers of oxidative stress in aquatic organisms (Armstrong, 2002). Antioxidant enzymes include: Cu/Zn superoxide dismutase (SOD) which are metalloenzymes that act as catalyzers of the dismutation of superoxide anion into O₂ and hydrogen peroxide (H₂O₂); Catalase (CAT) which reduces H₂O₂ to water in the peroxisomes while glutathione peroxidase (GPX) has the same function in cytosol. From these antioxidant enzymes, GPX activity is considered the most important one for measuring oxidative stress, since it is also the one that provides greater protection against this stress (Livingstone, 2003; Valavanidis et al., 2006).

Early studies with hypoxia actually considered ROS levels would be lower as the main substrate needed for ROS generation would be less available. However, such was not the case, as was determined in studies with mammals, showing ROS levels increasing when exposed to hypoxia (Abramov et al., 2007; Bell et al., 2007; Bickler et al., 2007). In non-mammals the same results seemed to occur, with an increase in ROS levels. Oxidative damage was observed in fish after hypoxia exposure and specifically in hypoxia-sensitive species (Lushchak et al., 2005; Behn et al., 2007)

Hyperoxia studies in fish focusing on pro-oxidant and antioxidant effects are relatively scarce (Ritola et al., 2000; Ritola et al., 2001; Livingstone et al., 2002; Welker et al., 2013). Nonetheless, effects on both antioxidant defenses and oxidative damage were observed. Continuous exposure to hyperoxigenation has been known to cause DNA stand-breaks in gills and activate hepatic glutathione-dependent defense systems in rainbow trout (Livingstone., 2001; Livingstone et al., 2002; Ritola et al., 2002), as well cause increases in lipid peroxidation and oxidized glutathione (Lushchak et al., 2006; Finne et al., 2008).

Activation of antioxidant defenses causes an overexpression of GPX, SOD and CAT mRNAs levels triggered by hyperoxia levels (Finne et al., 2008; Welker et al., 2013). mRNA levels provide an analysis of cell activity, with single mRNA expressions being useful as biomarkers of a particular stress in animals. The hyperoxia-induced regulation of gene expression in fish is still poorly known. A study made with Atlantic salmon exposed to slight hyperoxia (130% oxygen saturation) for a prolonged period of time showed that levels of hepatic CuZnSOD, catalase and GPX mRNAs had no significant differences when compared to the levels under normoxia (Olsvik et al., 2005). Rainbow trout hepatocytes maintained in hyperoxia conditions showed an increase of mRNA levels of GPX and SOD (Finne et al., 2008). Atlantic cod (*Gadus morhua* L.) also showed an increase in hepatic GPX mRNA levels after an exposition to hyperoxia (140% oxygen saturation) in comparison to the normoxia group (Olsvik et al., 2006). In a study with *Solea senegalensis*, there was an overexpression of hepatic antioxidant-related proteins after exposure to hyperoxia (223% O₂ saturation) (Salas-Leiton et al., 2009).

Scope of thesis

Indeed, few studies have been developed to completely understand the full consequences of hypoxia and hyperoxia on the immune system of fish as well as how affected is the specific gene expression of a possible biomarker of oxidative stress in Senegalese sole (Glutathione peroxidase), with no study being focused on both these subjects in this particular species. While hypoxia is known to affect fish in a negative way, there are still many uncertainties on how fish may actually cope with hyperoxia and to what extent, especially at the immune system level. Thus, the aim of this work is to give further insights on Senegalese sole's immune system, gene expression, gill morphology and cellular status following hyperoxia exposure, being fish submitted to different O₂ saturations ranging from mild hypoxia to O₂ supersaturation.

Material and Methods

Experimental design

The trial was performed at the Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) facilities (Porto, Portugal). Healthy Senegalese sole juveniles (30 ± 4.65 g wet weight) were obtained from a commercial fish farm, located in north-west Portugal (Aquacria, Aveiro, Portugal). Fish were maintained in recirculating aerated sea water according to standard protocols, with daily control of water parameters in a stock tank. The water was kept around 20°C, salinity at 24 ppm and dissolved oxygen at 96%. The fish were kept at a photoperiod of 12 hours of light and 12 hours of darkness. Fish were fed once a day to apparent satiety. Necessary cleanings were frequently done as well as daily parameter control of the water with control of the ammonia and nitrite levels, and in case these levels were not appropriate the proper adjusts would be made, such as doing regular water changes. Prior to each experiment, fish were removed from the stock tank and distributed among 18 L tanks filled with 8 L of seawater. The same daily procedures described above were performed in these experimental tanks to guarantee acclimatization. These tanks were all connected to a sump having constant water flux, with filtration being obtained through mechanical and biological filters. Six fish were assigned to each tank and exposed to different levels of dissolved oxygen. Mild hypoxia was established at 80% (negative control) while 2 hyperoxic conditions were tested (150, 200%). Fish reared under normoxic conditions (100% dissolved oxygen) served as positive control. Following 4 and 24 hours fish were removed from the tanks and sampled to assess gill damage, hematological (total and differential blood cell counts, hematocrit and hemoglobin) and innate immune (lysozyme, peroxidase) parameters as well as bactericidal activity using *Photobacterium damsela* subsp. *piscicida* (*Phdp*) strain PP3 (Costas et al., 2011; Costas et al., 2013a; Costas et al., 2013b). Sampled head-kidney tissue was posteriorly used for analysis of gene expression.

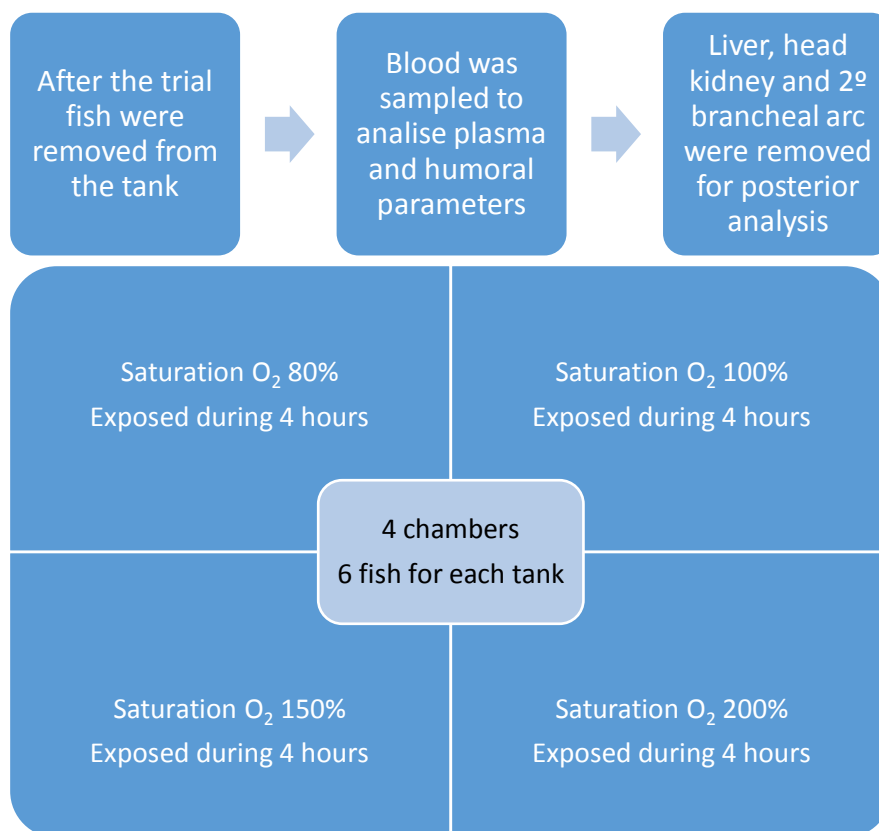


Figure 5 – Diagram explaining the experimental design. The same procedure was developed for the 24 hours trial.

To achieve an oxygen saturation level of 80% in the tank, all air-stones were removed during the trial (the level of oxygen was stable and controlled using an oxygen analyser). To achieve normoxia, two air-stones were used in each tank, and the same oxygen analyser was used to control the O₂ level. The supersaturation was obtained by injecting O₂ gas to the experimental tanks using an oxygen cylinder. To adjust the supersaturation level within the chambers, an oxygen regulator and an analyzer were used (Oxy-Reg, Loligo Systems, Denmark) - (Figure 6). The valve from the O₂ cylinder would open or close every single time the level of oxygen dropped or increased, to maintain the supersaturation level stable in the tank. This was achieved thanks to a solenoid valve that served as an intermediary between the oxygen cylinder and the Oxy-Reg analyser, and was connected to both devices. As soon as the oxygen level dropped below the saturation level needed for the trial, the solenoid valve would be opened by the Oxy-Reg analyser, allowing pumping of O₂ from the cylinder to the tank. As soon as the oxygen saturation increased to the pretended level, the solenoid valve would receive orders to close from the Oxy-Reg analyser, stopping the O₂ release into the tank.

For a constant and more precise monitorization of the O_2 saturation, a temperature compensated, oxygen meter named Fibox-3 Trace Device (Figure 7) was used at the same time together with the Oxy-Reg analyser to be absolutely sure the correct amount of O_2 was being released into the tank. An oxygen-sensitive optic fiber minisensor was placed in the tank to read the O_2 saturation, with values being collected by the Fibox-3 Trace Device and in turn transmitted directly to the PC (Figure 8). Fibox-3 Trace software was used to register the values in a 1-second interval. This way, O_2 level never deviated much from the saturation necessary for the respective trials, and was kept always at a stable level.



Figure 6 – Oxy-Reg analyzer. Source: <http://www.loligosystems.com>



Figure 7 – Fibox-3 Trace Device. Source: <http://www.presens.de>

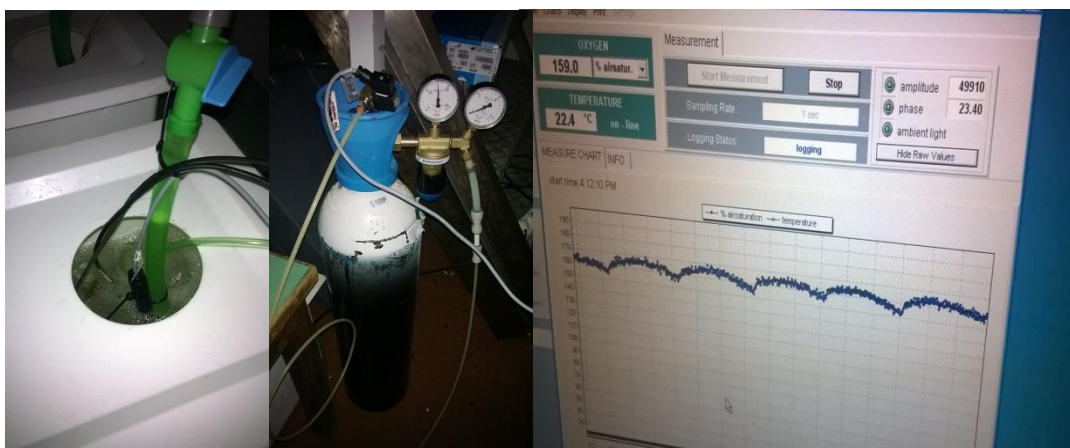


Figure 8 – Mounted system: In the left image, chamber shown with probes measuring the O_2 saturation as well as temperature; in the middle image, it is shown the cylinder used for pumping O_2 into the chambers; in the right image, values of O_2 saturation being registered by the Fibox-3 Trace Device are shown in the graphic.

Hematological procedure

After sampling the blood from the fish an analysis of the blood was made, involving these parameters:

- 1) Hematocrit, which is defined as packed cell volume or erythrocyte volume fraction is the volume (expressed in percentage) of red blood cells present at the total volume of blood. Basically, one fills a capillary tube with blood and then covers one of its ends with plasticine. Then these capillary tubes are placed in a hematocrit centrifuge during 10 minutes. After these 10 minutes, using a graphic reader, it is determined the percentage of red blood cells in the volume of blood, defined as RBC.



Figure 9 – Display of capillary tubes and hematocrit centrifuge in the left image (Source: <http://www.hawksley.co.uk>) and hematocrit chart in the right image.

- 2) Hemoglobin was then determined using the Drabkin colorimetric method (SPINREACT, ref.:1001230, Spain), with the following indices indicating information about the size of red blood cells and hemoglobin content.
- 3) Mean corpuscular volume (MCV) is the average size of a red blood cell:

$$(\text{Hematocrit (\%)} / \text{RBC (} 10^6 \mu\text{l)}) \times 10$$
- 4) Mean corpuscular hemoglobin (MCH) is the average amount of hemoglobin distributed for each red blood cell: $(\text{Hemoglobin (g/dl)} / \text{RBC (} 10^6 \mu\text{l)}) \times 10$
- 5) Mean corpuscular hemoglobin concentration (MCHC) is the average concentration of hemoglobin for each unit volume of red blood cells: $\text{Hemoglobin (g/dl)} / \text{Hematocrit (\%)} \times 100$
- 6) For the counting of red blood cells (RBC) and white blood cells (WBC) 2 solutions were prepared: for the white blood cells the solution was prepared from a dilution of 1/20 of homogenized blood in Hank's balanced salt solution mixed with heparin

(10 units/ml); for the RBC the solution was prepared mixing a dilution of 1/200 of homogenized blood in Hank's salt solution with heparin at the exact same concentration used for the WBC. After these solutions were prepared the counting of the cells was made using an optic microscope and a Neubauer chamber. As the concentrations of both solutions were different, WBC results are provided in the concentration of $10^4/\mu\text{l}$ and RBC are presented in $10^6/\mu\text{l}$.

7) Preparation and counting of blood smears:

After the blood extraction from the fish was made, a blood smear was prepared by placing a drop of homogenized blood in one of the ends of the slide, and then, using another slide, spreading the entire drop through the slide. It was then left to dry. Afterwards, the smear was stained with Wright's stain, fixation occurred during 1 minute using formol-ethanol (10% of formol and 90% of ethanol). After that a technique named Antonow's was executed, with the blood smear being left in the Antonow's stain for 15 minutes – this technique stains neutrophils as it detects the peroxidase activity performed by these cells (Afonso et al., 1998). The slides were counted at the microscope using the 100x objective. Oil immersion was necessary to observe the cells or else they would appear blurred, as oil immersion increases the resolution considerably. A total of 200 WBC were counted, with differentiation being made between these cells and catalogued under thrombocytes, lymphocytes, monocytes and neutrophils. Percentage and total concentration of each type of cell were determined after this procedure.

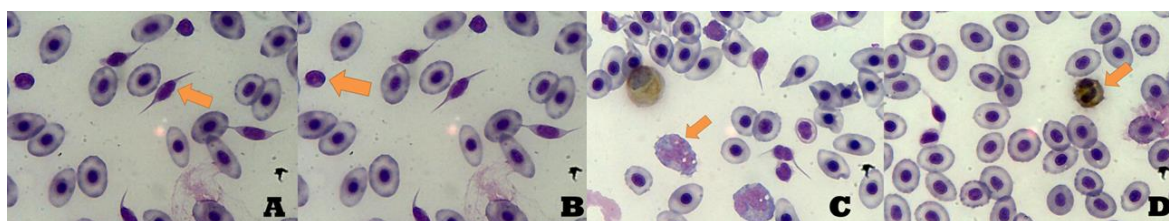


Figure 10 – During the microscope observation after the stain of the slide, these were the primarily identified white blood cells. Letter A corresponds to thrombocytes, B to lymphocytes, C to monocytes and D to neutrophils.

Humoral parameters analysis

Lysozyme –

Lysozyme activity was measured using a turbidimetric assay based on the method described by Ellis (1990) with some modifications (Wu et al., 2007). The turbidimetric method is used to determine the concentration of a substance in a solution. Measuring the loss in intensity of a light beam (with known wavelength) through a cuvette containing a solution with suspended particulate matter, a measurement is then given for the amount of absorbed light, that allows the determination of the substance concentration (Mary et al., 1994).

Using the samples collected, a standard bacterial suspension was added, and in turn read the absorbance in a spectrophotometer immediately after the addition of the bacteria and 4.5 minutes later. The difference found in the results is due to the bacterial lysis that occurs leading to a decrease in the number of bacteria present, and therefore less absorbance.

The solution was prepared by adding 0.05 M of sodium phosphate buffer (Na_2HPO_4) to 0.05 mg/mL^{-1} of *Micrococcus lysodeikticus*, with a pH of 6.2. After the solution was prepared, it was added to a microplate, and afterwards 15 μL of sampled plasma was added. Each well of the microplate was filled with a total of 265 μL of solution (15 μL from the plasma and 250 μL from the bacterial suspended solution), with triplicates being made for each sample. The absorbance was read at 450 nm in a Synergy HT microplate reader, 0.5 and 4.5 minutes later. For the determination of the amount of lysozyme present, a standard curve was made, since the equation of the curve is what allows the calculus of the lysozyme present in the plasma samples. This was achieved using Lyophilized hen egg white lysozyme and diluting it in 0.05 M of sodium phosphate buffer, once again in a pH of 6.2. Each solution had a decreasing amount of lysozyme present.

Peroxidase Activity -

For the determination of total peroxidase activity in plasma, the following procedure was executed:

15 μL of plasma serum was diluted in 135 μL of HBSS free of Ca^{2+} and Mg^{2+} in a 96-well plates. After that, 50 μL of 10 mM 3,3', 5,5'- tetramethylbenzidine hydrochloride (TMB; Sigma) as well as 50 μL of 5 mM H_2O_2 were added into the solution. A reaction occurred

immediately after the addition of these compounds, resulting in a color change. This reaction was stopped after 2 minutes as 50 µl of 2 M sulphuric acid (H₂SO₄) was added into the solution. The optical density was read at 450 nm in a Synergy HT microplate reader, Biotek. 150 µl of HBSS free of Ca²⁺ and Mg²⁺ were added to three wells to serve as blanks. Peroxidase activity was then measured having in account that one unit of peroxidase inflicts an absorbance change of 1 unit in the optic density (OD).

Bactericidal Activity –

Photobacterium damsela subsp. *piscicida* (*Phdp*) strain PP3 was used in the bactericidal activity assay. Bacteria were cultured for 48 h at 25 °C on tryptic soy agar (TSA; Difco Laboratories) and then inoculated into tryptic soy broth (TSB; Difco 21 Laboratories), both supplemented with NaCl to a final concentration of 1% (w/v). Bacteria in TSB medium were then cultured during 24h at the same temperature, with continuous shaking (100 rpm). Exponentially growing bacteria were collected by centrifugation at 3500 × *g* for 30 minutes, resuspended in sterile HBSS and adjusted to 1 × 10⁶ cfu ml⁻¹. Plating serial dilutions of the suspensions onto TSA plates and counting the number of cfu following incubation at 25 °C confirmed bacterial concentration of the inoculum.

In a round-bottom 96-well plate, in triplicates, 20 µl of plasma and 20 µl of *Phdp* were incubated for 2.5h at 25°C. Hank's balanced salt solution instead of plasma was used for positive control. To each well, was added 25 µl of MTT (3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide) and incubated for 10 minutes at 25°C to allow the formation of formazan. Plates were then centrifuged at 2000 × *g* for 10 min. The precipitate was dissolved in 200 µl of DMSO (dimethyl sulfoxide) and transferred to a flat-bottom 96-well plate. The absorbance of the dissolved formazan was recorded at 560 nm. Bactericidal capacity is calculated by comparison with the reference sample (positive control) and is expressed in percentage.

$$\% \text{ viable bacteria} = \frac{\text{Sample Abs.} \times 100}{\text{Abs. of the reference sample}}$$

$$\% \text{ non viable bacteria (bactericidal activity)} = 100 - \% \text{ viable bacteria}$$

Gill analysis

Gill sampling -

Samples were immediately fixed in phosphate buffered formalin (4%, pH 7.4) for 24h and subsequently transferred to ethanol (70%) until further processing.

Histology -

Gills samples were submerged in 1:50 w/v EDTA (0.5M; pH=7.8) for 5 days, to decalcify, and further processed and sectioned using standard histological techniques. Sections were stained with hematoxylin and eosin. Blinded evaluation was performed with particular attention to hyperplasia and /or cell hypertrophy, edema and telangiectases, abnormal vacuolization or gas bubbles in the primary lamellae, abnormal frequency of mucous and chloride cells, and infiltration of inflammatory cells. Three qualitative classes were established to classify the histomorphology of the gills: 1) normal, 2) with mild alterations and 3) with severe alterations.

Gene expression

RNA Extraction from head-kidney and gene expression analysis -

Total RNA was extracted from head-kidney slices using Trizol reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions and stored in 100 μ L RNase-free MilliQ H₂O. Genomic DNA was eliminated from the samples by DNase treatment according to the manufacturer's instructions (Grisp, Portugal). The RNA was stored at -80°C before further processing. The concentration and integrity of total RNA were assessed by measuring the absorbance at 260 nm and electrophoresis on 0.8% agarose gel, respectively. Total RNA (1 μ g) from each sample was reverse-transcribed using NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) following the manufacturer's instructions. The cDNA was then diluted in sterile distilled water (1:5 dilution), and the diluted cDNA preparation was used for semi-quantitative RT-PCR. The expression pattern was analyzed by semi-quantitative RT-PCR and the primer GPX1 was used for amplifying the GPX gene. The PCR condition consisted of a denaturation cycle of 94°C for 5 min, followed by 30 PCR cycles each consisting of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. Extension time in the last cycle was increased for 10 min. As an internal control, 18S mRNA was also amplified with 30 cycles by the primers of 18S-F and

18S-R. The PCR primers sequences used to quantify the mRNA levels of genes of interest are given in [table 1](#). The RNA sequences of 18S and GPX were obtained from the following GenBank accession numbers: EF126042 and HM068301, respectively. A 5 µL product of each PCR reaction was electrophoresed through 1.5% agarose gel and stained with Green Safe (NZYtech, Lisbon, Portugal) and then detected under UV light. Multi-gauge Fujifilm was used for gel analysis.

Gene	Forward Primer	Reverse Primer	Product length (bp)
18S	AGTGTGTGTCTACCCCTTCGC	TTATGACCCGCGCTTACTGG	120
GPX	TGCACACCCCTTGTTGTCT	GGGCTGACCAGGAATTCTC	150

Table 1 – Nucleotide sequences of PCR primers used for semi-quantitative RT-PCR. One housekeeping gene (18S) was run and used for the calculation of mean normalized expression.

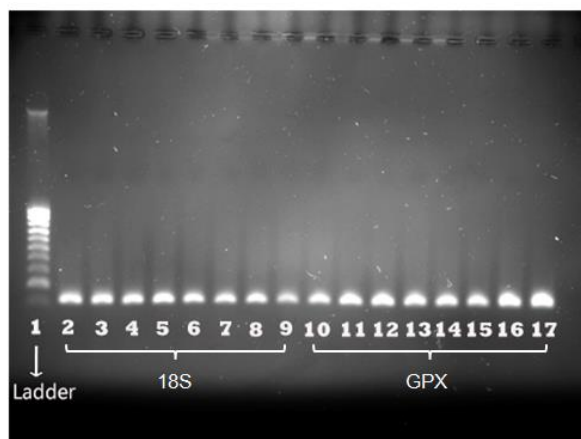


Figure 11 – Agarose gel from PCR reaction detected under UV light

Statistical analysis

Statistical analysis was made using the computer program Statistica 12 for Windows, with data being analyzed for normality and homogeneity of variance using Levene's test, before proceeding with a two-way ANOVA test (since the data analysis was made for two parameters, Time and Saturation). In case it was needed, data would be transformed for posterior statistical analysis. Tukey's test was used to assert significant differences between the different saturations as well as between the four and twenty-four hours trials. The level of significance was of $P \leq 0.05$ for the tests made, and all results provided in tables and graphs are showed in means and standard deviation (means \pm SD).

Results

Table 2 presents the absolute values of the hematological analysis. Hemoglobin and RBC levels increased significantly with the exposition time from 4 h to 24 h, for all saturations, while MCV decreased significantly with the exposition time from 4 h to 24 h, for all saturations.

Hematocrit and Hemoglobin levels showed significant differences between oxygen saturations, regardless of the exposure time. Hematocrit levels were significantly higher for fish reared at 80% when compared to 100% and 150% saturation. Hemoglobin levels also increased significantly in fish reared at 200% saturation, when compared to 80% and 100% saturation.

MCV, MCH and MCHC remained unchanged among different oxygen saturations and exposure time, while RBC and WBC did present significant differences in the interaction effect saturation x time. RBC increased significantly in fish reared at 200% saturation, from 4 hours to 24 hours exposition. Also, for the 24 hours exposition time, fish reared at 80% and 200% saturation showed a significant increase in RBC when compared to the control (100%). Regarding WBC, for the 4 hours exposition, a significant decrease was observed from 80% and 100% saturation to 150% saturation. For the 24 hours exposition, a significant decrease of WBC occurred from 80% and 100% saturation to 200% saturation.

Table 3 presents the absolute values of the different WBC and thrombocytes. Both WBC and thrombocytes showed significant differences among oxygen saturations, regardless of the exposure time. Both 150% and 200% saturation had overall the lowest concentration of these cell types, the only exception being for neutrophils with the lowest values being registered in the control group.

The interaction between saturation and time was significantly different for all cell types. Thrombocytes, for the 24 hours exposition, decreased significantly in fish reared at the 200% saturation when comparing to the 80% and 100% saturation. Lymphocyte levels presented a significant decrease in fish reared at 200% saturation, from the 4 hours exposition to the 24 hours exposition. Moreover, for the 24 hours exposition, a significant decrease of lymphocytes was observed in fish reared at 200% saturation when comparing with all the other saturations.

Monocyte levels decreased significantly with the exposition time from 4 h to 24 h, for all saturations. Also, monocytes presented a general decrease in the 150% saturation when compared to the control, after a 4h exposition. For the 24h exposition, monocyte concentration was significantly higher for the control and 80% saturation than 150% and 200%. Significant differences occurred for the same saturation at different exposition times, with the 200% saturation presenting monopenia in the 24h exposition when compared with the 4h exposition.

Finally, neutrophils concentration showed a significant increase in the 200% saturation in relation with the control and 150% saturation, after a 4h exposition. For the 24h exposition, a significant increase of neutrophils concentration was observed in the 80% saturation when compared to the 200% saturation. When comparing values of the same saturation but different exposition time, a significant decrease of neutrophils occurred in fish exposed to 200% saturation, from 4h to 24h of exposition.

Table 2. Hematocrit, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cells (RBC) and white blood cells (WBC) in Senegalese sole exposed to different O₂ saturations during 4 and 24 hours.

Parameters		Oxygen saturation							
		Hypoxia (80%)		Normoxia (100%)		Hyperoxia (150%)		Hyperoxia (200%)	
		4 hours	24 hours	4 hours	24 hours	4 hours	24 hours	4 hours	24 hours
Hematocrit	(%)	13.66 ± 2.31	14.45 ± 3.51	12.25 ± 2.21	12.25 ± 2.20	13.09 ± 2.98	11.82 ± 2.68	13.82 ± 2.32	14.67 ± 2.25
Hemoglobin	(g dl)	4.48 ± 0.65	4.67 ± 0.67	4.33 ± 0.22	4.98 ± 0.59	4.87 ± 0.44	4.91 ± 0.01	5.02 ± 0.37	5.19 ± 0.24
MCV	(μm ³)	16.12 ± 7.20	13.94 ± 3.89	14.70 ± 3.25	14.39 ± 2.88	14.35 ± 5.50	11.12 ± 2.06	17.48 ± 5.69	10.83 ± 2.01
MCH	(pg cell ⁻¹)	5.41 ± 3.29	5.59 ± 3.73	5.21 ± 1.01	6.05 ± 1.71	5.99 ± 2.29	4.86 ± 1.49	6.35 ± 1.82	3.89 ± 0.76
MCHC	(g 100 ml ⁻¹)	29.23 ± 10.89	38.92 ± 17.44	36.40 ± 7.56	41.66 ± 7.80	43.00 ± 14.69	43.41 ± 8.80	37.13 ± 5.86	36.06 ± 5.08
RBC	(×10 ⁶ μl)	9.72 ± 3.60	11.03 ± 0.34 ^a	8.58 ± 1.77	9.18 ± 3.13 ^b	9.95 ± 3.15	10.46 ± 3.44 ^{ab}	8.18 ± 2.72*	14.02 ± 3.93 ^a
WBC	(×10 ⁴ μl)	13.03 ± 4.76 ^a	13.00 ± 3.67 ^a	11.9 ± 4.60 ^a	12.9 ± 3.09 ^a	7.80 ± 1.33 ^b	9.20 ± 3.80 ^{ab}	9.50 ± 3.70 ^{ab}	4.50 ± 1.07 ^b

Two-way ANOVA

Parameters	Time	Saturation	Time × Saturation	Hypoxia (80%)	Normoxia (100%)	Hyperoxia (150%)	Hyperoxia (200%)
Hematocrit	ns	0.004	ns	a	b	b	ab
Hemoglobin	0.02	0.009	ns	b	b	ab	a
MCV	0.006	ns	ns	-	-	-	-
MCH	ns	ns	ns	-	-	-	-
MCHC	ns	ns	ns	-	-	-	-
RBC	0.002	<0.001	0.028	-	-	-	-
WBC	ns	<0.001	0.015	-	-	-	-

Presented values correspond to mean and standard deviation (means ± SD), respectively. Different letters determine that significant differences existed among saturations for the same time of exposition. The asterisk means that significant differences occurred for the same saturation at different times (2-way ANOVA, P ≤ 0.05; n = 12).

Table 3. Concentration of Thrombocytes, Lymphocytes, Monocytes and Neutrophils from Senegalese sole exposed to different O₂ saturations after 4 and 24 hours exposition, respectively.

Parameters	Oxygen saturation							
	Hypoxia (80%)		Normoxia (100%)		Hyperoxia (150%)		Hyperoxia (200%)	
	4 hours	24 hours	4 hours	24 hours	4 hours	24 hours	4 hours	24 hours
Thrombocytes ($\times 10^4 \mu\text{l}$)	4.23 \pm 2.02	5.39 \pm 1.75 ^a	4.55 \pm 1.79	5.07 \pm 1.37 ^a	2.73 \pm 1.55	3.60 \pm 1.71 ^{ab}	3.77 \pm 1.98	2.81 \pm 1.43 ^b
Lymphocytes ($\times 10^4 \mu\text{l}$)	7.68 \pm 4.27	5.91 \pm 3.09 ^a	6.93 \pm 2.92	7.35 \pm 2.80 ^a	4.83 \pm 1.39	5.77 \pm 3.55 ^a	5.16 \pm 2.21*	2.17 \pm 0.62 ^b
Monocytes ($\times 10^4 \mu\text{l}$)	0.84 \pm 0.44 ^{ab}	0.81 \pm 0.35 ^a	1.28 \pm 0.41 ^a	1.26 \pm 0.86 ^a	0.66 \pm 0.30 ^b	0.38 \pm 0.14 ^b	0.76 \pm 0.32 ^{ab*}	0.17 \pm 0.12 ^b
Neutrophils ($\times 10^4 \mu\text{l}$)	0.28 \pm 0.16 ^{ab}	0.40 \pm 0.27 ^a	0.15 \pm 0.11 ^b	0.30 \pm 0.28 ^{ab}	0.18 \pm 0.14 ^b	0.27 \pm 0.24 ^{ab}	0.66 \pm 0.56 ^{a*}	0.05 \pm 0.05 ^b

Two-way ANOVA

Parameters	Time	Saturation	Time \times Saturation	Hypoxia (80%)	Normoxia (100%)	Hyperoxia (150%)	Hyperoxia (200%)
Thrombocytes ($\times 10^4 \mu\text{l}$)	ns	<0.001	0.045	-	-	-	-
Lymphocytes ($\times 10^4 \mu\text{l}$)	0.010	<0.001	0.002	-	-	-	-
Monocytes ($\times 10^4 \mu\text{l}$)	0.007	<0.001	0.009	-	-	-	-
Neutrophils ($\times 10^4 \mu\text{l}$)	ns	0.041	<0.001	-	-	-	-

Presented values correspond to mean and standard deviation (means \pm SD), respectively. Different letters determine that significant differences existed among saturations for the same time of exposition. The asterisk means that significant differences occurred for the same saturation at different times (2-way ANOVA, $P \leq 0.05$; $n = 12$).

No significant differences were observed in the lysozyme (Figure 12) and peroxidase (Figure 13) activities, although a slight increase was denoted in the 24 hours trial for the 200% saturation when compared with the remaining saturations.

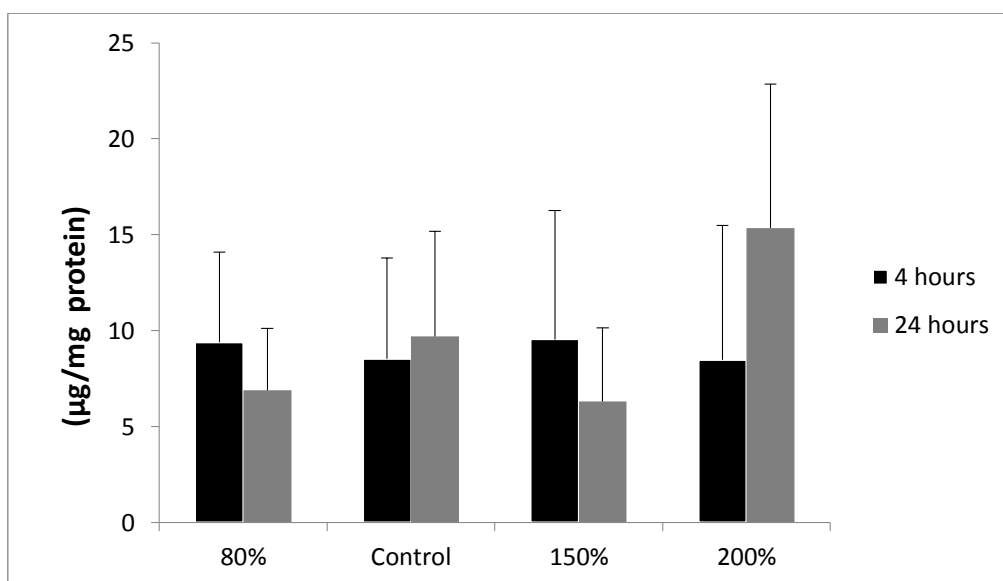


Figure 12 – Lysozyme activity of Senegalese sole for different O₂ saturations at a 4 hours and 24 hours period

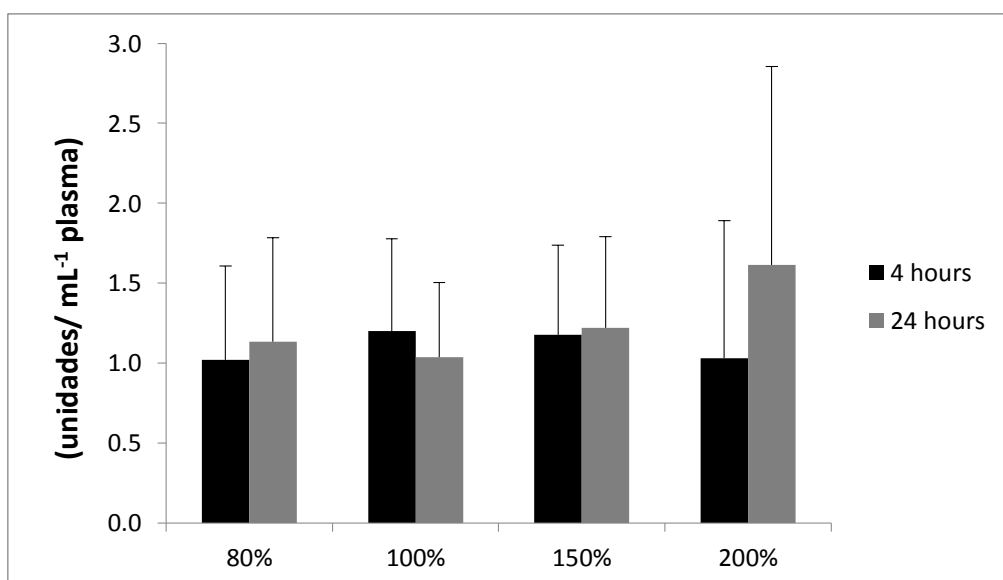


Figure 13 - Peroxidase activity of Senegalese sole for different O₂ saturations at a 4 hours and 24 hours period

No significant differences were observed for the bactericidal activity (Figure 14) at different O₂ saturations and time of exposition. There was a slight increase of activity at the 80% saturation when compared with other saturations at the 4 hours and 24 hours trial.

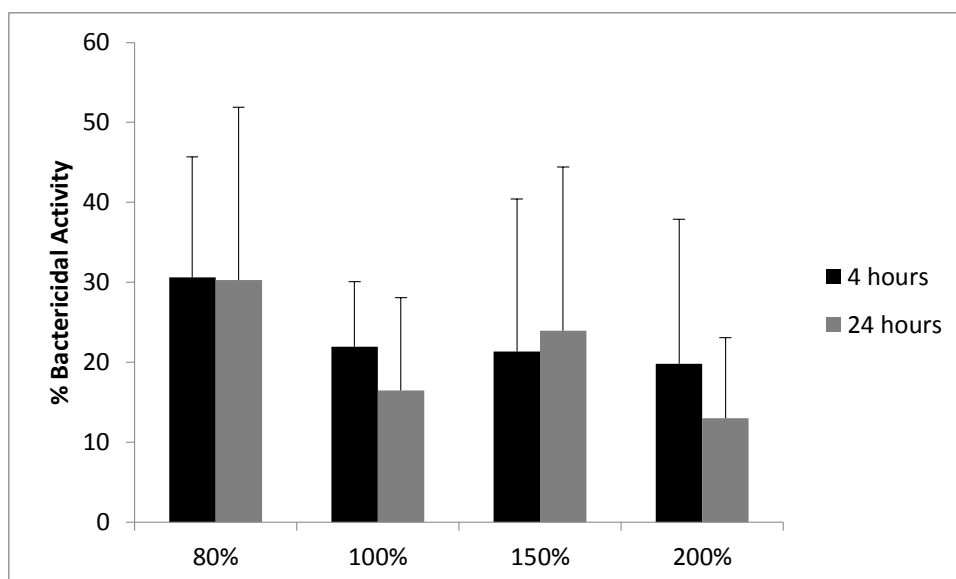


Figure 14- Bactericidal activity of Senegalese sole for different O₂ saturations at a 4 hours and 24 hours period

No significant differences were observed for the GPX expression at different O₂ saturations and time of exposition (Figure 15). There was a slight upregulation of GPX in the 80% saturation, for both exposition times and the same upregulation happened in the 200% saturation after a 24h exposition.

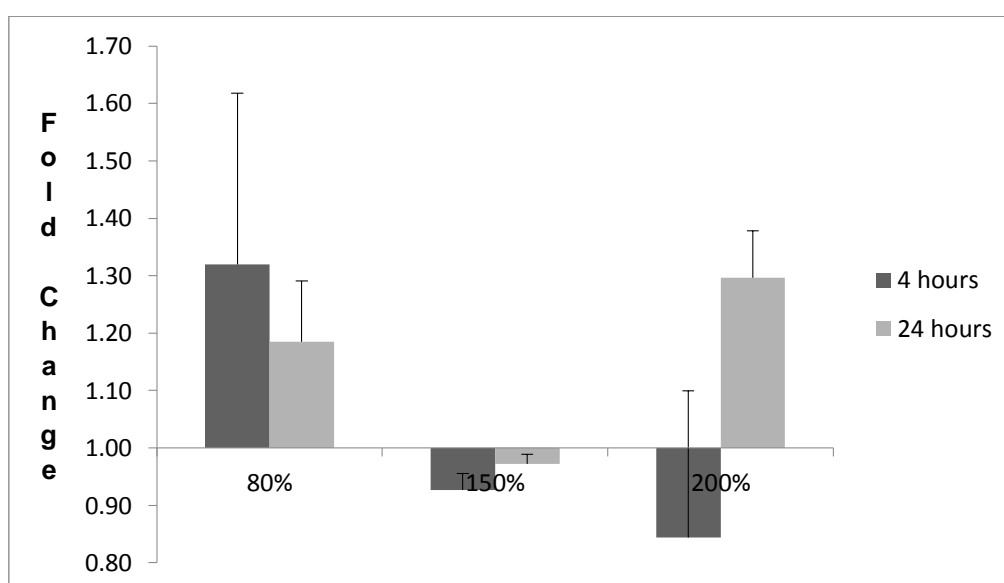


Figure 15- GPX expression of Senegalese sole for different O₂ saturations at a 4 hours and 24 hours period.

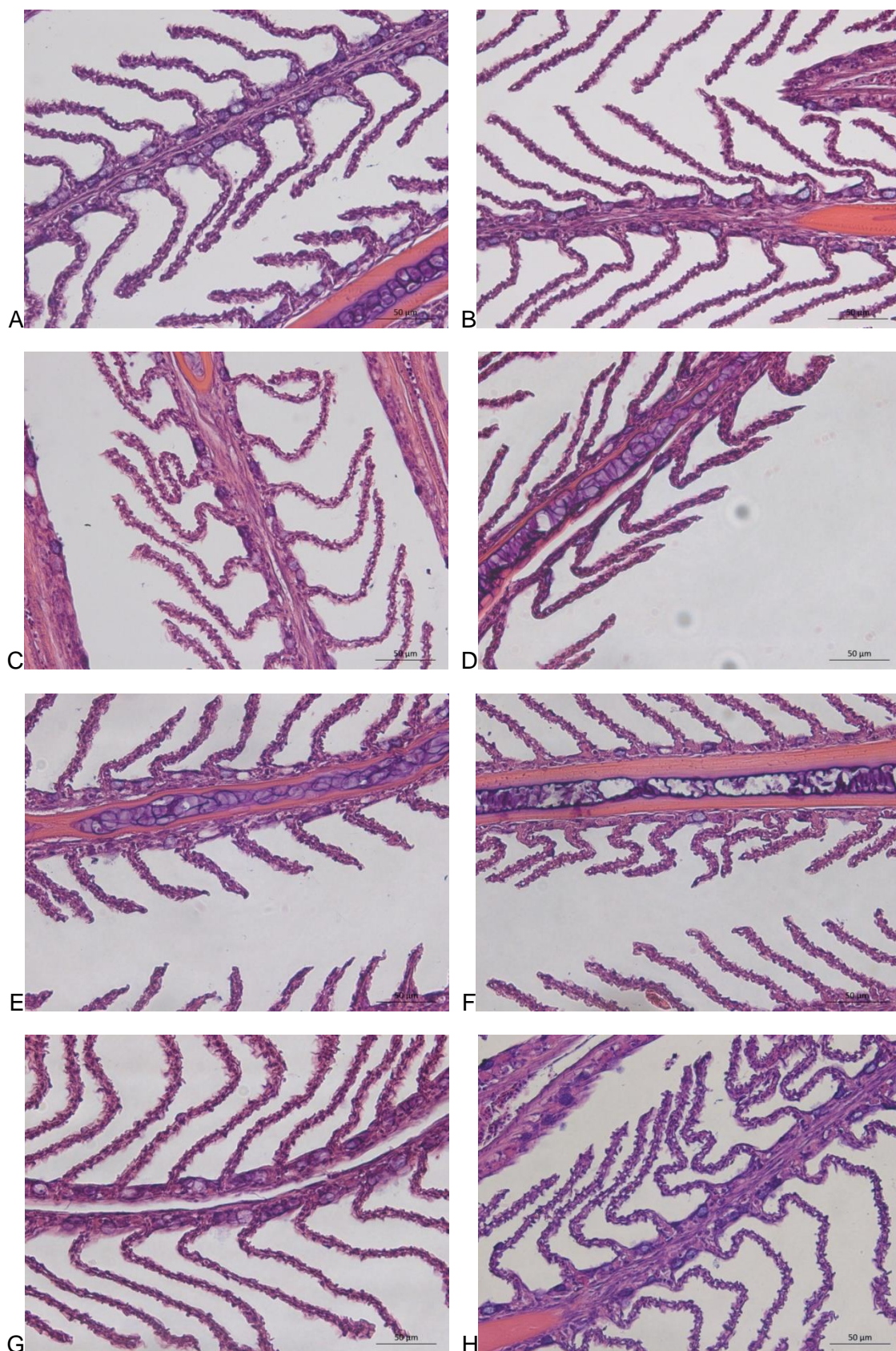


Figure 16 – Gills from sampled fish exposed to 80% (A, B), 100% (C, D), 150% (E, F) and 200% (G, H) O₂, for 4 (A, C, E, G) and 24h (B, D, F, H).

Regarding figure 16, no signs of abnormal cell growth or proliferation were present and vascularization was normal. The primary and secondary lamellae were well defined with scattered mucous cells along the edges, mainly in the primary lamellae. Secondary lamellae squamous epithelium was thin and adherent. No infiltration of inflammatory cells or edema was observed. Gills histomorphology was similar between experimental groups and no effects of acute hypoxia or hyperoxia were observed.

Discussion

O₂ hyper saturation has been described as being considered a stressor for fish maintained in an environment such as in intensive aquaculture production, and like many stressors may possibly induce immune system imbalances, increased susceptibility to diseases and even increased mortality (Ritola et al., 2002; Fridell et al., 2007; Thorarensen et al. 2010).

There seems to be no risk for Senegalese sole survival under conditions of O₂ supersaturation at least up to 200%, as no mortalities occurred to a maximum exposition of 24 hours. This is in agreement with studies made with hyperoxia in juvenile turbot and Atlantic salmon parr, since turbot showed only edemas at the end of few branchial lamellae after a 24h exposition to 120 and 150% oxygen saturation (Wu, 2014) and even survived exposure to 350% saturation for 10 days (Person-Le Ruyet, 2002); Atlantic salmon parr also presented no mortalities when reared at 150 and 175% saturation even though changes were noticed in their behaviour (vertical distribution and schooling) and in the cortisol levels. Other studies for rainbow trout and eel showed that these species cannot survive hyperbaric oxygenation for more than a few hours (5-15h at 2 ATA, absolute atmosphere of oxygen), as the gill surface begins suffering alterations in only 90 minutes exposition (Barthelemy et al., 1981; Sebert et al., 1984). To truly infer if Senegalese sole can adapt well to O₂ supersaturation, more parameters need to be analyzed, as well as increase the exposition time, since no mortalities occurred for periods up to 24 hours of exposition, but that may not be the case for longer exposition times.

Previous studies of the effects of hyperoxia in fish have determined some primary causes for a reduction in hematocrit due to exposition to hyperoxia values up to 180% saturation, as there may be an increase in plasma volume, decrease in number of erythrocytes or erythrocyte shrinkage observed immediately after the first 24 hours of

exposition, as was stated in previous studies with rainbow trout and Atlantic Salmon (Caldwell and Hinshaw, 1994; Ritola et al., 2002; Dabrowski et al., 2004; Hosfeld et al., 2010). These alterations occur as an adaptation to higher than normal oxygen-rich waters, where the need for oxygen transportation is reduced (Edsall and Smith, 1990, Hosfeld et al., 2010). However, no significant decrease was observed for RBC, MCV, MCH and MCHC when under mild hyperoxia exposure. In fact, regarding the hematocrit levels, the results obtained in this study indicate that 150% and 200% saturation showed no differences compared to normoxia. The higher value of hematocrit for the 80% saturation (moderate hypoxia) in comparison with the control, is explained by a need to acquire more O₂ and increase gas transport capacity, as fish can use several physiological mechanisms to compensate the reduced oxygen uptake, such as increasing breathing frequency, RBC, hemoglobin and Ht concentration (Perry and Gilmour, 2002; Wu et al., 2014).

RBC levels increased significantly for the 80% saturation compared to normoxia. This increase is explained by a recruitment of RBC needed to improve the oxygen transport capacity and help acquire more O₂ from waters with low levels of oxygen (Wu et al., 2014; Perry and Gilmour, 2002). The same case occurred for the 200% saturation as RBC and hemoglobin increased significantly for the 24h exposition compared to controls, going against previous studies made with rainbow trout, Atlantic salmon and Nile tilapia, where after mild hyperoxia exposure, erythrocytes decreased or showed no significant concentration differences when compared to normoxia, after a 12-24h period (Caldwell and Hinshaw, 1994; Ritola et al., 2002; Dabrowski et al., 2004; Hosfeld et al., 2010). A decrease of erythrocytes was expected for the 200% saturation, as the need for oxygen transport would decrease with rich oxygen waters, and a recruitment of erythrocytes would not be needed (Edsall and Smith, 1990; Hosfeld et al., 2010). However, such decrease in erythrocytes did not occur in this study. Also, the increase in hemoglobin level with exposition time for all saturations can be explained by the accompanied increase of RBC with exposition time, for all saturations. This increase in RBC with time for all saturations is probably due to an acclimation process to the overall oxygen changes, with fish striving to reach stable RBC levels.

WBC decreased significantly for the 150% O₂ saturation after 4h of exposition, when compared to normoxia and mild hypoxia. In particular, circulating monocyte levels were significantly lower for this saturation and exposition time when compared with the control, while other WBC showed no alterations for the same saturation and exposition time when compared with the control. Regarding the 24h exposition to this saturation, only monocyte levels seemed to be affected showing a significant decrease compared to the control. As for the 200% O₂ saturation, WBC and thrombocytes showed a significant

decrease when compared to normoxia and mild hypoxia, in the 24h exposition. This can be followed up with the results obtained for the lymphocyte and monocyte levels in the 200% saturation for the same exposition time, since the lowest levels were also registered for this saturation. In the case of neutrophils, for this saturation and exposition time, the significant decrease only existed when compared with mild hypoxia. When comparing exposition times, the 24h exposition to 200% saturation registered significant decreases in lymphocytes, monocytes and neutrophils when compared with the 4h exposition to the same saturation.

Studies regarding alterations to the immune system of fish after exposition to hyperoxia are rather limited, since those focused mainly on morphological and other physiological alterations (Fridell et al., 2008; Hosfeld et al., 2011). As a consequence, a lack of information seems to persist about the influence of hyperoxia in the immune status of fish. Nonetheless, hyperoxia has been identified as a possible stressor for fish, depending on factors such as saturation level, species resilience and its adaptations to oxygen-rich waters (Espmark et al., 2010; Tort, 2011; Welker et al., 2013). In this particular study, hyperoxia may have acted as an acute stressor due to the short exposition time. Without further analyses on corticosteroids levels as well as secondary stress responses, inferences are difficult to make over the severity of hyperoxia as a stressor. Regarding the results obtained, hyperoxia seems to affect Senegalese sole immune system, with a decrease in WBC for the 150% and 200% saturation having been observed. The greatest changes seem to have occurred for the 200% saturation after a 24h period, with an overall decrease in thrombocytes and all the WBC analyzed in this study.

Several works dealing with leucocytes distribution have shown stress as a major influence in changes to cell numbers and traffic patterns, with differences in the leucocyte distribution in different body compartments being observed (Ortuño et al., 2001; Costas et al., 2011). Acute stress response results in an increase in circulating leucocyte numbers, with a mobilization of blood cells (both erythrocytes and white cells) taking part due to the acute response (Verburg-van Kemenade et al., 2000; Costas et al., 2013; Costas et al., 2014). The changes in blood leucocyte numbers are normally characterized by an increase in numbers of neutrophils and a decrease in lymphocytes and monocytes numbers. Such case may have happened for the 150% saturation after a 4h period, with a decrease in monocytes when compared to the control, even though lymphocytes and neutrophils did not show any differences with the control. A similar situation may have happened for the 200% saturation after a 24h period, since both lymphocytes and monocytes decreased in the blood when compared to control, as a possible result of

mobilization due to an acute response, although neutrophils did show similar levels when compared with normoxia and even decreased in numbers compared to mild hypoxia. To further emphasize this, the mobilization would be more intensified with prolonged time, which did happen in this study as lymphocytes and monocytes decreased significantly in fish after an exposure of 24h to 200% saturation in comparison with an exposure of only 4h.

Regarding lysozyme and peroxidase activities, results usually vary depending on the species and type of stressor, with different studies showing contradictory results. In some of those studies lysozyme activity decreased (Olsen et al., 1993; Cnaani and McLean, 2009), while in other studies the activity significantly increased in stressed individuals (Rotllant et al., 1997; Caipang et al., 2009). For this study, no significant differences existed among saturations. Furthermore, bactericidal activity did not show any significant deviation in the comparison made between all saturations, suggesting immune stimulation probably was not very intensified. The absence of an activation factor, such as bacterial infection may however contribute to explain this lack of response of lysozyme and peroxidase as the production of lysozyme is enhanced in response to stimulation relatively to non-stimulated specimens (Ellis, 2001).

As for mild hypoxia (80% saturation), it seems no negative effects occurred for the immune system, since it had no differences in the WBC count and immune parameters, when compared with normoxia. It is possible Senegalese sole is capable of enduring a suboptimal level of oxygen of at least 80% saturation for a period up to 24 hours, without consequences to the immune system.

A variety of external factors are known to alter the morphology of the gill epithelium in fish, with oxygen availability being included as one of these factors (Tzaneva et al., 2011). In a recent study, gill remodeling occurred in crucian carp and goldfish in response to temperature and O₂ (approximately 300% O₂ saturation) (Tzaneva et al., 2011), the main feature observed being the proliferation of an intralamellar cell mass that embedded the lamellae in a mass of cells aiming at protecting the tissue from oxidative damage. In sturgeon (*Huso huso*), 8 weeks exposure to hyperoxygenation conditions (115% O₂ saturation) resulted in clubbing of the secondary lamellae, edema in the basal membrane of secondary lamellae, hyperplasia, congestion of the secondary lamellae, telangiectases and excessive vacuolization in the base of the secondary lamellae (Lakani et al., 2013). All the above described features are suggested to decrease respiratory surface and, thus, prevent or at least diminish oxidative damage. The abnormal vacuolization may be due to the excessive oxygen saturation that may conduct to gas bubble disease (Lakani et al.,

2013). However, in both studies described above, exposures to abnormal oxygenation conditions were much longer than in the present one. Thus, acute exposure (24h) of Senegalese sole to mild hypoxia or hyperoxia conditions had no effects in gills histomorphology.

An adequate oxygen concentration is needed for the survival of fish, however with the exposure to extreme oxygen conditions such as hypoxia and hyperoxia, these may provoke serious damages in the DNA, possibly influencing the expression of certain genes (Liepelt et al., 1995). Damage is caused by ROS that originate from the partial reduction of oxygen into energy and water. These ROS, if left unchecked may cause oxidative stress in fish. Species that normally are faced with oxygen limiting conditions in their environment need to implement a defensive strategy to counter this. Strategies mostly involve changes in tissue-specific activities of antioxidant enzymes (Lushchak et al., 2001; Lushchak et al., 2005; Lushchak and Bagnyukova, 2006). Antioxidant enzymes along with low molecular weight scavengers are synthesized to protect the aquatic organism from these ROS. Most of the experiments that were made to assess the effects of oxygen levels were mostly done in hypoxia conditions, but studies that focused on hyperoxygenation show an elevated activity of several antioxidant enzymes such as CAT, SOD and GPX (Ritola et al., 2002; Finne et al., 2008; Welker et al., 2013; Costas et al., 2015). These alterations in the antioxidant defenses are clearly important to maintain ROS levels at adequate levels in conditions where ROS production is expected to increase as it is clearly the case in hyperoxia conditions (Welker et al., 2013). GPX activity is mostly used as an indicator of oxidative stress, as this enzyme is regarded as the most important one in the protection against this stress (Livingstone, 2001; Valavanidis et al., 2006). Hyperoxia has been reported to cause elevated levels of hepatic GPX after an exposition of 14 days to 173% O₂ saturation in rainbow trout (Ritola et al., 2002), while in another study a more extended period of hyperoxia (126 days; 130% O₂ saturation) caused no changes in antioxidant enzyme expression (including GPX) in Atlantic salmon (Olsvik et al., 2005). According to the results obtained in this study, GPX gene expression showed no significant differences among saturations in comparison with the control, for both exposition times. It may be that simply there was no significant exposure to hyperoxia to cause a generation of ROS in the fish and consequently cause significant oxidative stress. It is also possible that GPX expression may be a limited marker of oxidative stress induced by hyperoxia, since other studies did not come to any conclusion after using GPX expression as a biomarker of oxidative stress or the physiological adaptations did not reflect in the transcriptome.

Conclusion

Hyperoxia has been proven to have several consequences in fish, ranging from positive to negative effects, depending of the exposition period and O₂ saturation. In this study, mild and high hyperoxia appear to influence the hematological and immune status of fish, with most significant changes observed at the highest saturation tested (200%). A peak of hemoglobin concentration and RBC numbers occurred for this saturation. As for the immune system, a decrease in WBC was observed in both tested hyperoxia saturations, probably due to leucocyte distribution to different body compartments as a reaction to hyperoxia, suggesting some degree of immunostimulation. This study further suggests that hyperoxia may have acted as an acute stressor mobilizing WBC as a result of an acute stress response. In contrast to cell migration, humoral immune parameters suggest there was no activation of the innate immune response. Regarding expression of GPX, it is possible hyperoxia did not induce a high enough level of oxidative stress to augment the synthesis of GPX. Still, further studies with increasing exposition times and other O₂ saturation levels may bring more insights on how hyperoxic conditions may affect fish welfare.

References

- Acerete, L., Balasch, L.C., Espinosa, E., Josa, A., Tort, L., (2004). Physiological responses in Eurasian perch (*Perca fluviatilis*, L.) subjected to stress by transport and handling. *Aquaculture* 237, 167–178.
- Abramov, A.Y., Scorziello, A., Duchen, M.R., 2007. Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. *J. Neurosci.* 27, 1129–1138.
- Afonso, A., Lousada, S., Silva, J., Ellis, A.E., Silva, M.T. (1998) Neutrophil and macrophage responses to inflammation in the peritoneal cavity of rainbow trout *Oncorhynchus mykiss*. A light and electron microscopic cytochemical study. *Dis Aquat Organ.* 34 (1). p. 27-37.
- Anderson, R. S., Brubacher, L. L., Calvo, L. R., Unger, M. A. & Bureson, E. M. (1998). Effects of tributyltin and hypoxia on the progression of *Perkinsus marinus* infections and host defence mechanisms in oyster, *Crassostrea virginica* (Gmelin). *Journal of Fish Diseases* 21, 371–379.
- Anguís V., Cañavate J.P., (2005). Spawning of captive Senegal sole (*Solea senegalensis*) under a naturally fluctuating temperature regime. *Aquaculture* 243, 133–145.
- Aragão, C., Corte-Real, J., Costas, B., Dinis, M.T., Conceição, L.E.C., (2008). Stress response and changes in amino acid requirements in Senegalese sole *Solea senegalensis* Kaup 1758. *Amino Acids* 34, 143–148.
- Armstrong, D., 2002. Oxidative stress, biomarkers and antioxidant protocols. In: Walker, J.M. (Ed.), *Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp. 1– 322.
- Barthelemy, L., Belaud, A., Chastel, C., (1981). A comparative study of O₂ toxicity in vertebrates. *Respir. Physiol.* 44, 261-268.
- Bickler, P.E., Buck, L.T., 2007. Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. *Annu. Rev. Physiol.* 69, 145–170.

Behn, C., Araneda, O.F., Llanos, A.J., Celedon, G., Gonzalez, G., 2007. Hypoxia-related lipid peroxidation: evidences, implications and approaches. *Respir. Physiol. Neurobiol.* 158, 143–150.

Bell, E.L., Klimova, T.A., Eisenbart, J., Schumacker, P.T., Chandel, N.S., 2007. Mitochondrial reactive oxygen species trigger hypoxia-inducible factor-dependent extension of the replicative life span during hypoxia. *Mol. Cell. Biol.* 27, 5737–5745.

Bergheim A., Martin G., Anders N., Per M., Holland, Per Krogedal and Viv Crampton. (2005). A newly developed oxygen injection system for cage farms. *Aquacultural Engineering* 34, 40-46.

Boleza, K.A., Burnett, L.E., Burnett, K.G. (2001) Hypercapnic hypoxia compromises bactericidal activity of fish anterior kidney cells against opportunistic environmental pathogens. *Fish and Shellfish Immunology* 11, 593-610.

Bols, N.C., L. Brubacher, R. Ganassin and L.E.J. Lee (2001): Ecotoxicology and innate immunity of fish. *Dev. Comp. Immunol.*, 25, 853-873.

Bowden, T. (2008). Modulation of the immune system of fish by their environment. *Fish and shellfish immunology* 25, 373-383.

Brauner, C.J., Thorarensen, H., Gallagher, P., Farrell, A.P., and Randall, D.J. (2000). CO₂ transport and excretion in rainbow trout (*Oncorhynchus mykiss*) during graded sustained exercise. *Respir. Physiol.* 119, 69-82.

Bunch, E.C. & Bejerano, I. (1997). The effects of environmental factors on the susceptibility of hybrid tilapia *Oreochromis niloticus* x *Oreochromis aureus* to *Stertococcosis*. *Israeli Journal of Aquaculture* 49, 67–76.)

Bustin, S.A. (Ed.), 2004. A–Z of Quantitative PCR. International University Line, La Jolla, pp. 1 –882.

Caldwell, C.A., Hinshaw, J., (1994). Physiological and haematological responses in rainbow trout subjected to supplemental dissolved oxygen in fish culture. *Aquaculture* 126, 183–193.

Caldwell A.C., Hinshaw J. (1995) Tolerance of rainbow trout to dissolved oxygen supplementation and a *Yersinia ruckeri* challenge. *J Aquat Anim Health* 7, 168–171.

Caipang, C.M.A., Berg, I., Brinchmann, M.F., Kiron, V. (2009) Short-term crowding stress in Atlantic cod, *Gadus morhua* L. modulates the humoral immune response. *Aquaculture* 295, 110-115.

Cnaani, A., McLean, E. (2009) Time-course response of cobia (*Rachycentron canadum*) to acute stress. *Aquaculture* 289, 140-142.

Colt J., White R., (Eds.) (1991) Water quality considerations and criteria for high density fish culture with supplemental oxygen. *Fisheries Bioengineering Symposium 10*, American Fisheries Society, Bethesda, Maryland, USA , pp. 372–385.

Costas B., Aragão C., Mancera J.M., Dinis M.T., Conceição L.E.C. (2008) High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole *Solea senegalensis* (Kaup 1858) juveniles. *Aquaculture Research* 39, 1–9.

Costas B., Conceição L.E.C, Aragão C., Martos J.A., Ruiz-Jarabo I., Mancera J.M., Afonso A. (2011) Physiological responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) after stress challenge: effects on non-specific immune parameters, plasma free amino acids and energy metabolism. *Aquaculture* 316, 68–76.

Costas B., Rêgo P.C.N.P., Simões I., Marques J.F., Castro-Cunha M., Afonso A. (2013a) Cellular and humoral immune responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) following challenge with two *Photobacterium damsela* subsp. *piscicida* strains from different geographical origins. *Journal of Fish Diseases* 36, 543-553.

Costas B., Aragão C., Dias J., Afonso A., Conceição L.E.C. (2013b) Interactive effects of a high quality protein diet and high stocking density on the stress response and some innate immune parameters of Senegalese sole *Solea senegalensis*. *Fish Physiology and Biochemistry* 39, 1141-1151.

Crampton, V., Holland, P.M., Bergheim, A., Gausen, M., Naess, A., (2003). Oxygen effects on caged salmon. *Fish Farming International*, June, 26-27.

Dalmo RA, Ingebrigsten K, Bogwald J., (1997). Non-specific defense mechanisms in fish, with particular reference to the reticuloendothelial system (RES). *J Fish Dis.* 20, 241-73.

Dabrowski, K., Lee, K.J., Guz, L., Verlhac, V., Gabaudan, J., 2004. Effects of dietary ascorbic acid on oxygen stress (hypoxia or hyperoxia), growth and tissue vitamin concentrations in juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 233, 383–392.

Dejours, P. (1977). The effect of hyperoxia on the breathing of marine fishes. *Comp. Biochem. Physiol.* 58A, 409-411.

Dinis, M.T., (1986). Quatre Soleidae de l'Estuaire du Tage. Reproduction et Croissance. Essai d'Élevage de *Solea senegalensis* Kaup 1858. Thèse d'état ès-Sciences Naturelles, Université de Bretagne Occidentale, France.

Dinis, M.T., Reis, J., Arrobas, I., (1996). Evaluation of the farming potential for *Solea senegalensis* Kaup, a new species for aquaculture in the Mediterranean area. *Book of Abstracts World Aquaculture 96*, Bangkok, 107–109.

Dinis, M.T., Ribeiro, L., Soares, F. and Sarasquete, C. (1999). A review on the cultivation potential of *Solea senegalensis* in Spain and Portugal. *Aquaculture* 176, 27-38.

Drake, P., Arias, A.M., (1989). Hábitos alimentarios de estados juveniles de peces en los caños de la bahía de Cádiz (SO de España). In: Yufera, M. (Ed.), *Aquicultura Intermareal*. Instituto de Ciencias Marinas de Andalucía-CSIC, Cádiz, Spain, pp. 249–257.

Edsall, D.A., Smith, C.E., (1990). Performance of rainbow trout and Snake River cutthroat trout reared in oxygen-supersaturated water. *Aquaculture* 90, 251–259.

Ellis, A.E. (2001). *Innate host defense mechanisms of fish against viruses and bacteria*. *Developmental and Comparative Immunology*. 25(8-9), 827-839.

Evans DL., Jaso-Friedmann L. (1992). Nonspecific cytotoxic cells as effectors of immunity in fish. *Ann Rev Fish Dis* 2, 109-121.

Espmark A.M. & Baeverfjord G. (2010) Effects of hyperoxia on behavioural and physiological variables in farmed Atlantic salmon (*Salmo salar*) parr. *Aquaculture International* 17, 341–353.

Esteban, M.A., Muñoz, J., Meseguer, J. (2000). Blood cells of sea bass (*Dicentrarchus labrax* L.). Flow cytometric and microscopic studies. *The Anatomical Record*. 258, 80–89.

FAO. (2014). The State of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations.

Feldman, G. M., Dannenberg, A. M. & Seed, J. L. (1990). Physiologic oxygen tensions limit oxidant-mediated killing of schistosome eggs by inflammatory cells and isolated granulomas. *Journal of Leukocyte Biology* 47, 344–354.

Finne, E.F., Olsvik, P.A., Berntssen, M.H.G., Hylland, K., Tollefsen, K.E., 2008. The partial pressure of oxygen affects biomarkers of oxidative stress in cultured rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Toxicol Vitro* 22, 1657–1661.

Foss, A., Evensen, T.H., Øiestad, V., (2002). Effects of hypoxia and hyperoxia on growth and food conversion efficiency in the spotted wolffish *Anarhichas minor* (Olafsen). *Aquaculture Res.* 33, 437–444.

Foss, A., Tollen, T., & Øiestad, V. (2003). Growth and oxygen consumption in normal and O₂ supersaturated water, and interactive effects of O₂ saturation and ammonia on growth in spotted wolffish (*Anarhichas minor* Olafsen). *Aquaculture*, 224, 105–116.

Fridell, F., Gadan, K., Sundh, H., Taranger, G.L., Glette, J., Olsen, R.E., Sundell, K., Evensen, Ø., (2007). Effect of hyperoxygenation and low water flow on the primary stress response and susceptibility of Atlantic salmon *Salmo salar* L. to experimental challenge with IPN virus. *Aquaculture* 270, 23–35.

García López A. (2006). Efectos del fotoperiodo y del termoperiodo sobre el ciclo reproductor en cautividad del lenguado senegalês *Solea senegalensis* (Kaup 1858). PhD thesis, Universidad de Cádiz, Spain, p 310.

Goss, G. G., Laurent, P., & Perry, S. F. (1994). Gill morphology during hypercapnia in brown bullhead (*Ictalurus nebulosus*): role of chloride cells and pavement cells in acid–base regulation. *Journal of Fish Biology*, 45, 705– 718.

Gouveia, A. (2014). Production of farmed turbot and Senegalese sole in Portugal. *International Aquafeed* 17, 27-29.

Hosfeld, C.D., Engevik, A., Mollan, T., Lunde, T.M., Waagbø, R., Olsen, A.B., Breck, O., Stefansson, S., Fivelstad, S., (2008). Long-term separate and combined effects of environmental hypercapnia and hyperoxia in Atlantic salmon (*Salmo salar* L.) smolts. *Aquaculture* 280, 146–153.

Hosfeld, C.D., Handeland S., Fivelstad, S., Stefansson, S., (2010). Physiological effects of normbaric environmental hyperoxia on Atlantic salmon (*Salmo salar* L.) presmolts. *Aquaculture* 308, 28-33.

Hosfeld, C.D., Lemarie, G., Breuil, G., Fivelstad, S. (2011). Effects of hyperoxic water conditions under different total gas pressures in European Sea bass (*Dicentrarchus labrax*). *Aquaculture* 318, 191-198.

Imsland, A.K., Foss, A., Conceição, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema, P., White, P., (2003). A review of the culture potential of *Solea solea* and *Solea senegalensis*. Rev. Fish Biol. Fish. 13, 379 – 407.

Janeway, C.A., Travers, P., Walport, M., Shlomchik, M.J., (2005). Immunobiology: The Immune System in Health and Disease, 6th ed. Garland Press, New York and London.

JNICT (1990). Perfil Nacional Em Ciências Do Mar – Nível II Portugal. Junta Nacional De Investigação Científica e Tecnológica, Lisboa.

Kowaltowski, A.J., de Souza-Pinto, N.C., Castilho, R.F., Vercesi, A.E., 2009. Mitochondria and reactive oxygen species. Free Radic. Biol. Med. 47, 333–343.

Kregel, K.C., Zhang, H.J., 2007. An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. Am. J. Physiol. 292, R18–R36.

Lagardère, F., Decamps, P., Quero, J.-C., (1979). Découverte les long des costes de la Charente-Maritime d'une population de *Solea senegalensis* Kaup, 1858 (Soleidae, Pleuronectiformes). Ann. Soc. Sci. Nat. Charente-Maritime 6, 563–572.

Lakani, F.B., Sattari, M., Falahatkar, B. (2013) Effect of different oxygen levels on growth performance, stress response and oxygen consumption in two weight groups of great sturgeon *Huso huso*. Iranian journal of fisheries sciences 12, 533-549.

Livingstone DR (2003) Oxidative stress in aquatic organisms in relation to pollution and aquaculture. Revue Me'dVe't 154:427–430

Lygren, B., Hamre, K., Waagbø, R., (2000). Effect of induced hyperoxia on the antioxidant status of Atlantic salmon *Salmo salar* L. fed three different levels of dietary vitamin E. Aquaculture Res. 31, 401–407.

Lushchak, V.I., Bagnyukova, T.V., Lushchak, O.V., Storey, J.M., Storey, K.B., 2005. Hypoxia and recovery perturb free radical processes and antioxidant potential in common carp (*Cyprinus carpio*) tissues. Int. J. Biochem. Cell Biol. 37, 1319–1330. Martínez-Alvarez, R.M., Morales, A.E., Sanz, A., 2005. Antioxidant defenses in fish.

Mach, William J.; Thimmesch, Amanda R.; Pierce, J. Thomas; Pierce, Janet D. (2011). "Consequences of Hyperoxia and the Toxicity of Oxygen in the Lung". Nursing Research and Practice 1–7. doi:10.1155/2011/260482.

Magnadottir B., (2006). Innate immunity of fish (overview). *Fish Shellfish Immunology* 20,137-51.

Magnadottir B., (2010). Immunological Control of Fish Diseases. *Marine Biotechnology* 12, 361-379.

Manzano C. (1993) Aquaculture Development in Andalusia and Water Management Techniques in the Wetlands. In: *Aquaculture and Coastal Wetlands*. INIP, Lisbon, Portugal

Mary C. Haven, Gregory A. Tetrault, Jerald R. Schenken (1994). *Laboratory Instrumentation*. John Wiley and Sons. ISBN 0471285722.

Mazzini M., Scapigliati G., Abelli L., Terribili F. R., Scalia D., Fanelli M., Fausto A.M., Mastrolia L. e Romano N. (1998). Studio dell'ossigeno liquido sul Sistema immunitario dei pesci eurialini in acquacoltura intensiva/ A study on the effect of hyperoxygenation on the immune system of euryaline fish in aquaculture. *Biol.Mar.Medit.* 5(3), 1633-1641.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fish.* 9, 211–268.

Murray, C. K., Fletcher, T. C. (1976). The immunohistochemical localization of lysozyme in plaice (*Pleuronectes platessa*) tissue. *J. Fish. Biol.* 9, 329-334.

Muiswinkel, W. B., Nakao, M., (2014). A short history of research on immunity to infectious diseases in fish. *Developmental and Comparative Immunology.* 43, 130-150.

Neumann, N.F., Stafford, J.L., Barreda, D., Ainsworth, A.J., Belosevic, M. (2001). Antimicrobial mechanisms of fish phagocytes and their role in host defense. *Developmental and Comparative Immunology.* 25, 807-825.

Oliva-Teles, A. (2012). Nutrition and health of aquaculture fish. *Journal of fish diseases,* 35, 83-108.

Olmedo. M., Peleteiro J.B., Cal. R, Linares, F. (2003) Crecimiento de juveniles de lenguado (*Solea senegalensis*, Kaup 1858) en Galicia. In: *Actas IX Congreso Nacional de Acuicultura*. Junta de Andalucía, Cádiz, Spain, pp 383–385.

Olsen, Y.A., Reitan, L.J., Roed, K.H. (1993) Gill Na^+ , K^+ -atpase activity, plasma-cortisol level, and nonspecific immune-response in Atlantic salmon (*Salmo salar*) during parr-smolt transformation. *Journal of Fish Biology* 43, 559-573.

Olsvik, P. A., Kristensen, T., Waagbø, R., Rosseland, B. O., Tollefsen, K. E., Baeverfjord, G. and Berntssen, M. H. G. (2005). mRNA expression of antioxidant enzymes (SOD, CAT and GSH-Px) and lipid peroxidative stress in liver of Atlantic salmon (*Salmo salar*) exposed to hyperoxic water during smoltification. *Comp. Biochem. Physiol.* 141C, 314-323.

Olsvik, P. A., Kristensen, T., Waagbø, R., Rosseland, B. O., Tollefsen, K. E., Toften, H. (2006). Effects of hypo- and hyperoxia on transcription levels five stress genes and the glutathione system in liver of Atlantic cod *Gadus morhua*. *The journal of experimental biology* 209, 2893-2901.

Ortuño, J., Cuesta, A., Rodríguez, A., Esteban, M.A., Meseguer, J., (2002). Oral administration of yeast, *Saccharomyces cerevisiae*, enhances the cellular innate immune response of gilthead seabream (*Sparus aurata* L.). *Vet. Immunol. Immunopathol.* 85, 41–50.

Parkin. J. (2001). An overview of the immune system. *The Lancet* 357, 1777-1789.

Person-Le Ruyet, J., Delbard, C., Chartois, H. and Le Delliou, H. (1997). Toxicity of ammonia to turbot juveniles: I-effects on survival, growth and food utilization. *Aquat. Living Res.* 10, 307-314.

Person-Le Ruyet, J., Pichavant, K., Vacher, C., Le Bayon, N., Séverè, A., Boeuf, G., (2002). Effects of O₂ supersaturation on metabolism and growth in juvenile turbot (*Scophthalmus maximus* L.). *Aquaculture* 205, 373–383.

Person-Le Ruyet, J., Lacut, A., Le Bayon, N., Le Roux, A., Pichavant, K., Quémener, L., (2003). Effects of repeated hypoxic shocks on growth and metabolism of turbot juveniles. *Aquatic Living Resources* 16, 25-34.

Pousão-Ferreira P. (1993) Considerações sobre a piscicultura no Algarve. In: *Aquaculture and Coastal Wetlands*. INIP, Lisbon, Portugal

Pulsford, A.L., Crampe, M., Langston, A., Glynn, P.J., (1995). Modulatory effects of disease, stress, copper, TBT and vitamin E on the immune system of flatfish. *Fish Shellfish Immunol.* 5, 631– 643.

Randall, J.E. & R.H. Kuiter, (1982). Three New Labrid Fishes of the Genus *Coris* from the Western Pacific. *Pacific Science.* 36 (2), 159-173.

Randall, J.E. & Taylor, L.R., (1988). Review of the Indo-Pacific fishes of the serranid genus *Liopropoma*, with descriptions of seven new species. –Indo-Pacific Fishes 16, 1-47.

Ramos, J., (1982). Estudio de la edad y crecimiento del lenguado *Solea solea* (L. 1758). (Pisces Soleidae). Investig. Pesq. 46, 275–286.

Ramalho, A., Dinis., M.T. *Portuguese Aquaculture: current status and future perspectives*, in *Aquaculture Europe*. 2010.

Rauta, R.P., Nayak B., Das S., (2012). Immune system and immune responses in fish and their role in comparative immunity study: A model for higher organisms. Immunology Letters 148, 23-33.

Ritola, O., Tossavainen, K., Kiuru, T., Lindström-Seppä, P., Mölsä, H., (2002). Effects of continuous and episodic hyperoxia on stress and hepatic glutathione levels in one summer-old rainbow trout (*Oncorhynchus mykiss*). J. Appl. Ichthyol. 18, 159–164.

Roberts, R.J., Ellis, A.E. (2012). The Anatomy and Physiology of Teleosts. In: Roberts, R.J. (Ed.), *Fish Pathology*. Blackwell Publishing Ltd.

Rodriguez, R.B., (1984). Biología e cultivo de *Solea senegalensis* Kaup 1858 en Golfo de Cadiz. PhDThesis, Universidad de Sevilla, Spain.

Rodríguez, J.L., Souto, B.F., (2003) Engorde dellenguado senegalês (*Solea senegalensis* KAUP, 1858) en Galicia con temperatura controlada. In: IX Congreso Nacional de Acuicultura. Junta de Andalucía, Cádiz, Spain, pp 407–409

Rotllant, J., Pavlidis, M., Kentouri, M., Adad, M.E., Tort, L. (1997). Non-specific immune responses in the red porgy *Pagrus pagrus* after crowding stress. Aquaculture 156, 279-290.

Samuel, C.E., (1991). Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. Virology 183, 1-11.

Salas-Leiton, E., Cánovas-Conesa, B., Zerolo, R., López-Barea J., Cañavate J.P., Alhama, J., (2009). Proteomics of Juvenile Senegal Sole (*Solea senegalensis*) Affected by Gas Bubble Disease in Hyperoxygenated Ponds. Mar Biotechnol. 11, 473-487.

Sardinha, M., (2009). Aquaculture on a national context. European Aquaculture.

Sargolia, M., Terova-Saroglia, G., Knight, M., & Cecchini, S. (1995). Ruolo dell'iperossigenazione e della salinità su alcune performance di spigola (*Dicentrarchus labrax*, L.). *Atti XI Congresso Nazionale ASPA, Grado (GO)*, pp. 49–50.

Sebert, P., Barthelemy, L., Peyraud, C., (1984). Oxygen toxicity in trout at two seasons. *Comp. Biochem. Physiol.*, A 78, 719-722.

Secombes C.J. (1996). The nonspecific immune system: cellular defenses. In: Iwama G, Nakanishi T. editors. *The fish immune system*, Fish Physiology series vol. 15 San Diego: Academic press, p. 63-103.

Secombes C.J., Zou J. (2011). Teleost fish interferons and their role in immunity. *Developmental and Comparative Immunology* 35, 1376-1387.

Seppola M, Stenvik J, Steiro K, Solstad T, Robertsen B, Jensen I., (2007). Sequence and expression analysis of an interferon stimulated gene (ISG15) from Atlantic cod (*Gadus morhua* L.). *Developmental and Comparative Immunology* 31, 156-171

Thurston, R.V., C. Chakoumakos, and R.C. Russo. (1981). Effect of fluctuating exposures on the acute toxicity of ammonia to rainbow trout (*Salmo gairdneri*) and cutthroat trout (*S. clarki*). *Water Res.* 15, 911-917.

Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T., Vinci, B.J., (2001). *Recirculating Aquaculture Systems*. NRAC Publication, Cayuga Aqua Ventures, Ithaca.

Tort, L., (2011). *Stress and immune modulation in fish*. *Dev. Comp. Immunology*, 35, 1366-1375.

Tzaneva, V., Bailey, S., Perry, S.F., (2011) The interactive effects of hypoxemia, hyperoxia, and temperature on the gill morphology of goldfish (*Carassius auratus*). *American journal of physiology-regulatory integrative and comparative physiology* 300, R1344-R1351.

Valavanidis A, Vlahogianni T, Dassenakis M, Scoullos M (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol Environ Saf* 64,178–189.

Verburg-van Kemenade, B.M.L., Novak, B., Engelsma, M.Y. and Weyts, F.A.A. (1999) Differential effects of cortisol on apoptosis and proliferation of carp B-lymphocytes from head kidney, spleen and blood. *Fish Shellfish Immunol.* 9, 405-415.

Verburg-van Kemenade, B.M.L., Stolte, E.H., Metz, J.R., Chadzinska, M., (2009). Neuroendocrine-immune interactions in teleost fish. In: Bernier, N.J., Van DerKraak, G., Farrell, A.P., Brauner, C.J. (Eds.), *Fish Physiology*, vol. 28. Academic Press Inc., San Diego, USA, pp. 313–364.

Vogelbein, W. K., Zwerner, D. E., Kator, H., Rhodes, M. & Cardinal, J. (1999). Epizootic mycobacteriosis in Chesapeake Bay striped bass. In *Proceedings of the 24th Annual Eastern Fish Health Workshop*, 8–11 March 1999, Atlantic Beach, NC. West Virginia: National Fish Health Research Laboratory.

Wajsbrodt, N. et al., (1991). Acute toxicity of ammonia to juvenile gilthead seabream *Sparus aurata* under reduced oxygen levels. *Aquaculture*, 92, 277–288

Wang, T., Lefevre, S., Huong, D.T.T., Cong, N.V., Bayley, M., (2009). Effects of hypoxia on growth and digestion. In: Richards, J., Brauner, C.J., Farrell, A.P. (Eds.), *Fish Physiology*, Vol. 27. Academic Press, San Diego, CA, 361–396.

Watts M., Munday B.L., Burke C.M. (2001). Immune responses of teleost fish. *Australian Veterinary Journal* 79, 570–574.

Wedemeyer GA (1997). Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In: Iwama GK, Pickering AD, Sumpter JP, Schreck CB (eds) *Fish stress and health in aquaculture*. Cambridge University Press, Cambridge, pp 35–71.

Welker, T. L., L. Chhorn, Y. A. Mediha and H. K. Klesius. (2007). Growth, immune function, and disease and stress resistance of juvenile Nile tilapia (*Oreochromis niloticus*) fed graded levels of bovine lactoferrin. *Aquaculture* 262, 156–162.

White, K., O'Neill, B., Tzankova, Z. (2004). At a Crossroads: Will Aquaculture Fulfill the Promise of the Blue Revolution? *SeaWeb Aquaculture Clearinghouse*, pp. 17.

Whyte, S.K. (2007). The innate immune response of finfish – A review of current knowledge. *Fish & Shellfish Immunology* 23, 1127–1151.

Wu, R.S.S. and N.Y.S. Woo. (1984). Respiratory responses and tolerance to hypoxia in two marine teleosts, *Epinephelus akaara* (Temminck and Schlegel) and *Mylio macrocephalus* (Basilewsky). *Hydrobiologia*. 119, 209–217.

Wu, Z., You, F., Wen A., Ma D., Zhang P. (2014). Physiological and morphological effects of severe hypoxia, hypoxia and hyperoxia in juvenile turbot (*Scophthalmus maximus* L.). Aquaculture Research 1-9.

Yano T., (1996). The nonspecific immune system: humoral defense. In: Iwama G, Nakanashi T, editors. The fish immune system, Fish Physiology series vol.15 San Diego, CA: Academic Press, 106-57.

<http://www.hawksley.co.uk>

<http://www.loligosystems.com>

<http://www.presens.de>